STAIN TECHNOLOGY

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PROGRESS IN THE STANDARDIZATION OF STAINS

TESTS TO WHICH STAINS ARE SUBMITTED BEFORE CERTIFICATION

It was in 1923—twenty years ago—that the first stain (methylene blue) was put on the certification basis. Today practically all the most commonly used stains (about 50 in number) are sold in this country almost wholly under one of the labels indicating certification by the Stain Commission. Bottles so labeled are being used wherever in the world American armed forces are located; and presumably—thanks to Lend-Lease—in the camps and base hospitals of our Allies, as well. The majority of today's laboratory workers are comparatively young, and their experience in such work hardly goes back twenty years. Most of them take Commission certification for granted and do not realize what it stands for. They certainly do not appreciate what their predecessors in the first World War had to contend with because of the unstandardized, and sometimes mislabeled, dyes which had been hastily prepared by an inexperienced dye industry and were *supposed* to be suitable for biological staining.

The Commission on Standardization of Biological Stains was organized in 1922 under the auspices of the National Research Council with the object of improving and standardizing the unreliable stains on the market in the post-war period. Another object was to make this country independent of Europe as a source of these very necessary tools of the biologist. The present war has proved that the second object was decidedly worth while.

The Commission has assisted the National Formulary Committee of the American Pharmaceutical Association in drawing up specifications of biological stains; and as a result, the National Formulary now requires Commission certification in the case of all stains that have been put on the certification basis. The specifications in the National Formulary have been adopted by the Army and Navy for use in the procurement of supplies. The result is that the armed forces of the United States now require Commission certification on biological stains.

The Stain Commission has thus acquired a semi-official nature, altho it is neither Government sponsored nor Government supported,

nor is it part of any national society having official recognition, like the American Medical Association, American Pharmaceutical Association, or the American Public Health Association—all of which organizations, by the way, are represented on the Executive Committee of the Commission.

As a result there are today more laboratory workers than ever before who are using certified stains. These technicians may well be interested in the Commission label on packages of stains which they use and may want to know what that label means. What does the label indicate as to the uses to which stains that bear it can safely be put? In answer to this question, the following list of certified stains has been prepared and after each stain is listed the tests made by the Commission. This means that each batch of stain certified has been tested and found satisfactory in the listed procedures, and may be safely assumed to perform equally well in a variety of other related methods.

Acid Fuchsin

Determination of dye content and spectrophotometric absorption ratio; especially to determine conformity with National Formulary specifications.
Tests to determine suitability for use in Andrade indicator, and as to performance in the Mallory and VanGieson connective tissue stains.

Aniline blue W.S.

Determination of approximate dye strength and spectrophotometric absorption ratio; especially to determine conformity with National Formulary specifications.
Tests to determine degree of decolorization obtainable by adding normal NaOH.
Tests as to performance in Mallory's connective tissue stain.

Alizarin red S

Determination of spectrophotometric absorption ratio.
Tests for staining of bone.

Auramin O

Determination of dye content and spectrophotometric absorption ratio.
Tests as to performance in fluorescence technic for acid-fast bacilli.

Azocarmine G

Determination of dye content.
Tests as to performance in Heidenhain's modification of Mallory's connective tissue stain.

Azure A

Determination of dye content and spectrophotometric absorption ratio.
Tests as to performance in the Haynes modification of French's tissue stain, and as a constituent of MacNeal's Tetrachrome Stain.

Azure B

Determination of dye content and spectrophotometric absorption ratio.
Tests as a component of the Giemsa Stain for malaria parasites.

Azure C

Determination of spectrophotometric ratio.
Tests as to performance in French's tissue stain.

Basic Fuchsin

Determination of dye content and spectrophotometric absorption ratio; especially to determine conformity with National Formulary specifications.
Tests as to bacteriostatic action, and as to performance in the Ziehl-Neelson technic for the tubercle organism, in the Feulgen technic, and in the Endo medium.

Bismarck Brown

Determination of dye content and spectrophotometric absorption ratio; especially to determine conformity with National Formulary specifications.
Tests as a counterstain for animal tissue, and in Neisser's diphtheria stain.

Brilliant Cresyl Blue

Determination of dye content and spectrophotometric absorption ratio; especially to determine conformity with National Formulary specifications.
Tests as to performance in supravital staining.

Brilliant Green

Determination of dye content and spectrophotometric absorption ratio; especially to determine conformity with National Formulary specifications.
Tests as to bacteriostatic action, and as to performance in brilliant green bile media for the colon-typhoid group of bacteria.

Carmine

Tests as to performance in Schneider's aceto-carmine technic for plant cytology, and in Mayer's alcoholic acid carmine for animal tissue.

Chlorazol Black E

Determination of spectrophotometric absorption ratio.
Tests as to performance in general histological and cytological work.

Congo Red

Determination of dye content and spectrophotometric absorption ratio; especially to determine conformity with National Formulary specifications.
Tests as to performance as a counterstain for animal tissue.

Cresyl Violet

Determination of dye content and spectrophotometric absorption ratio.
Tests as to performance in staining fresh tissue, especially that removed from tumors at biospy.

Crystal Violet

Determination of dye content and spectrophotometric absorption ratio; especially to determine conformity with National Formulary specifications.
Tests as to bacteriostatic action and as to performance in Flemming's triple staining technic, in the KI-crystal-violet technic, and in the Gram stain.

Ethyl Eosin

Determination of dye content and spectrophotometric absorption ratio; especially to determine conformity with National Formulary specifications.
Tests as to performance in Harris Stain for Negri bodies.

Eosin B

Determination of dye content.
Tests as to performance in Mallory's phloxine-methylene-blue stain, and in the hematoxylin-eosin procedure.

Eosin Y

Determination of dye content and spectrophotometric absorption ratio; especially to determine conformity with National formulary specifications.
Tests as to performance in the eosin-methylene-blue medium, as a counterstain to Heidenhain's and Delafield's hematoxylin, and in Wright's stain for blood.

Erythrosin

Determination of dye content and spectrophotometric absorption ratio.
Tests as to performance in Jackson's crystal violet stain.

Fast Green FCF

Determination of dye content and spectrophotometric absorption ratio.
Tests as a nuclear stain with safranin.

Giemsa stain

Tests as a stain for blood, especially for malarial parasites in thick films.

Hematoxylin

Tests as to performance in Heidenhain's, Delafield's, Weigert's, and Mayer's procedures.

Indigo Carmine

Determination of dye content and spectrophotometric absorption ratio.
Tests as a counterstain to carmine and to basic fuchsin.

Janus Green B

Determination of dye content and spectrophotometric absorption ratio.
Tests as to performance in supravital staining of blood.

Jenner's Stain

Tests as a blood stain.

Light Green SF yellowish

Determination of dye content and spectrophotometric absorption ratio.
Tests as a nuclear stain with safranin.

Malachite Green

Determination of dye content and spectrophotometric absorption ratio.
Tests as to performance in bacterial spore stains, and as a counterstain to safranin in plant histology.

Martius Yellow

Determination of dye content and spectrophotometric absorption ratio.
Tests as a counterstain to resorcin blue for staining pollen tubes.

Methyl Green

Determination of dye content and spectrophotometric absorption ratio; especially to determine conformity with National Formulary specifications.
Tests as a counterstain to Bismarck brown, and as a constituent of the Pappenheim-Saathoff stain for gonorrheal smears.

Methyl Orange

Determination of dye content and spectrophotometric absorption ratio.
Tests as a counterstain to crystal violet and to hematoxylin in animal and plant tissues.

Methyl violet 2B

Determination of dye content and spectrophotometric absorption ratio.
Tests as to performance in the Flemming triple stain, in the KI-crystal-violet stain, and in the Gram stain.

Methylene Blue Chloride

Determination of dye content and spectrophotometric absorption ratio; especially to determine conformity with National Formulary specifications.
Tests as to performance in the Mallory phloxine-methylene-blue technic for tissue, in Wright's stain for blood, also as a stain for bacteria in milk and for diphtheria organisms in smears.

Methylene Blue Thiocyanate

Tests as an indicator for reduction in milk.

Methylene Violet

Tests as a constituent of MacNeal's tetrachrome stain.

Neutral Red

Determination of dye content and spectrophotometric absorption ratio; especially to determine conformity with National Formulary specifications.
Tests as to performance in supravital staining of blood.

Nigrosin

Determination of spectrophotometric ratio.
Tests as to performance in Dorner's bacterial spore stain.

Nile Blue A

Determination of dye content and spectrophotometric absorption ratio.
Tests as to performance in the Smith and Mair technic as a differential fat stain.

Orange G

Determination of dye content and spectrophotometric absorption ratio; especially to determine conformity with National Formulary specifications.
Tests as to performance in the Mallory triple stain, in the KI-crystal-violet technic, and in Heidenhain's stain for animal tissue.

Orange II

Determination of dye content and spectrophotometric absorption ratio.
Tests as a counterstain to crystal violet and hematoxylin for plant and animal tissue.

Orcein

Tests as to performance in Mollier's quadruple tissue stain, and as a stain for elastin in tissue.

Phloxine

Determination of dye content and spectrophotometric absorption ratio.
Tests as to performance in Mallory phloxine-methylene-blue stain for tissue.

Pyronin

Determination of dye content and spectrophotometric absorption ratio; especially to determine conformity with National Formulary specifications.
Tests as to performance in Pappenheim-Saathof stain for gonorrheal smears.

Resazurin

Tests as an indicator for reduction in milk.

Rose Bengal

Determination of dye content and spectrophotometric absorption ratio; especially to determine conformity with National Formulary specifications.
Tests as a stain for soil bacteria.

Safranin O

Determination of dye content and spectrophotometric absorption ratio; especially to determine conformity with National Formulary specifications.
Tests as to performance in the Flemming triple stain, and in the Gram stain, and as a nuclear stain with light green or fast green.

Sudan III

Determination of dye content and spectrophotometric absorption ratio.
Tests as a fat stain.

Sudan IV

Determination of dye content and spectrophotometric absorption ratio; especially to determine conformity with National Formulary specifications.
Tests as a fat stain.

Tetrachrome Stain (MacNeal)

Tests as a blood stain.

Thionin

Determination of dye content and spectrophotometric absorption ratio.
Tests as to performance in Stoughton's stain for plant tissue.

Toluidine Blue

Determination of dye content and spectrophotometric absorption ratio; especially to determine conformity with National Formulary specifications.
Tests as to performance in the Haynes modification of French's tissue stain and as a stain for frozen sections.

Wright's Stain

Tests as a blood stain.

—H. J. Conn

LOCATING IODINE IN TISSUES AUTOGRAPHICALLY, ESPECIALLY AFTER FIXATION BY FREEZING AND DRYING

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ABSTRACT.—**The ability of radioactive elements to affect photographic emulsions enables the detection of radioactive iodine in the thyroid. By placing unstained histological sections of a thyroid (from an animal treated with radioactive iodine) in contact with the gelatin side of medium lantern slide plates, each accumulation of radioactive iodine in the section affects the photographic plate. After exposures prolonged for several days to several weeks depending on the amount of radioactivity in the tissues, the plate is developed and fixed by routine photographic methods. The histological section is stained and may be compared under the microscope to the reactions on the plate or "autographs".**

In an attempt to detect the location of the inorganic iodine which is displaced during fixation and embedding by ordinary methods because of its solubility, a simplified freezing-drying technic for fixation was devised which, at least with the thyroid, yielded well fixed sections. The quick freezing was obtained with acetone-dry-ice mixtures; and the drying was performed at –25° to –30°C. Preliminary addition of paraffin to the tube in which the drying was performed made possible the inclusion in vacuum by heating the tube when drying was completed. The tissue could then be sectioned at 10μ on the microtome. The slides were placed on photographic plates for detection of radioactive iodine as indicated above. Before staining, the sections were treated with absolute alcohol for denaturation of the proteins.

When a radioactive substance is present in the tissues, it is possible to locate it histologically by placing thin sections of the tissues on a sensitive photographic emulsion. In the case of radioactive iodine,

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we used the following technic derived from methods published by Lacassagne *et al.* (1924, 1925) for the detection of polonium, and by Hamilton *et al.* (1940) for that of radioactive iodine.

Both the isotope I^{130} with a half life of 12.6 hours, from the cyclotron of the University of Rochester, and the isotope I^{131} with a half life of 8 days prepared at the Massachusetts Institute of Technology, were used. The localization of radioactive iodine was investigated in the thyroids of animals (rats, dogs, guinea pigs, rabbits) given an intravenous injection of a single dose of radioactive iodine. The sections of the glands to be placed in contact with photographic emulsions were prepared either by routine histological technics or by a simplified freezing-drying technic.

In the former case, the tissues were fixed in Bouin's fluid, dehydrated in alcohol, put thru xylene and paraffin, and cut at 10μ. In case of the short-lived (12.5 hours) I^{130} isotope, these operations were speeded up, so that the paraffin sections were affixed to the slides from 6 to 8 hours after the animals had been sacrificed. The slides were then dried for 1 or 2 hours at 42°C., taken thru xylene, alcohol, and 0.5% celloidin, and left for 2 minutes in 70% alcohol. When dry, the sections were ready for deposition on the photographic emulsion.

In some cases, the freezing-drying technic of Gersh (1932) was used in order to eliminate liquid fixatives. This method enables one to retain the water-soluble components of the tissues (here inorganic iodine) in the position which they occupy during life. It was possible to obtain a satisfactory fixation, while simplifying the described technics to such a point that only normal histological equipment was required, with the addition of a Hyvac pump. Thyroids of rats weighing about 10 mg. or fragments of thyroids of similar size from rabbits, dogs or guinea pigs were used with equally good results. The living tissue was placed at the end of a strip of supple cardboard measuring 10 by ½ inch. The organ adhered to the cardboard and was immediately dropped into an acetone-dry-ice mixture for a few minutes. It should be noted, however, that the tissues happened to be left for a few hours in the freezing mixture, without apparent damage. Meanwhile about 20 ml. of hot pure paraffin (MP. 54–56°) were poured into a 75-ml. Pyrex test tube, where they were left to harden. Then the Pyrex test tube was half-immersed in a 5-liter thermos bottle containing a mixture of absolute alcohol and dry ice maintained between −25° and −30°C. The piece of cardboard holding the tissue in the acetone-dry-ice mixture was rapidly removed and held over the mouth of the Pyrex test tube. The end of the

cardboard with the tissue was clipped off and dropped in the Pyrex test tube, the air content of which was at a temperature of −25° to −30°. Finally the test tube was connected by means of a rubber stopper with a Hyvac pump in the body of which a vacuum had been previously established. The vacuum was turned on. With some practice the transfer of the tissue to the drier and the establishment of the low pressure may be made in a few seconds. Speed is imperative to prevent warming the tissues.

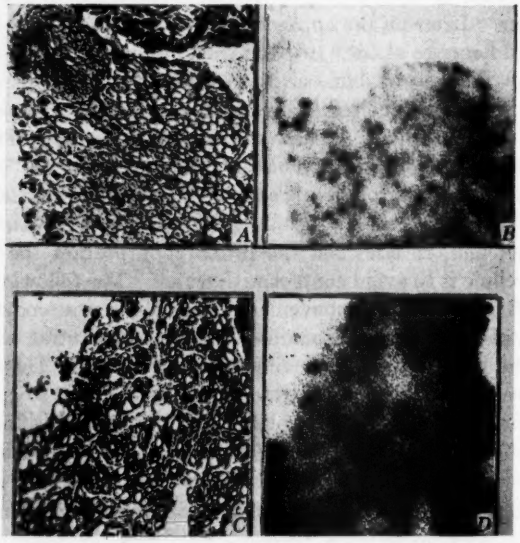
The operations were usually started in the morning. The temperature in the large thermos bottle was maintained between −25 and −30°C. for about 3 hours, by adding a few pieces of dry ice once every hour. Later on the apparatus was allowed to warm up slowly for several hours to about −10°, and then more rapidly to room temperature. With a low flame or in a 60° water bath the paraffin was carefully and slowly melted. The impregnation of the tissue by the paraffin was immediate. The vacuum was shut off. The tissue could then be sectioned at 10μ on the microtome.

The main difficulty of the technic was to affix the sections on the slides. No aqueous solution could be used since water disorganizes the tissue; and, in addition, the primary purpose of the freezing-drying technic is to avoid contact with water. The following process was found to be the most convenient. The slides were coated with a thin film of standard egg-albumin-glycerin (1:1) solution and left to dry on the hot plate for some time (15 to 30 min.). Then the sections were affixed by rapidly stretching a "tenia" of 6 to 10 sections over the slides kept on the hot plate (56° C.). The paraffin was not removed from the sections before placing the slides on the gelatin emulsion. It was therefore necessary to interpose a thin microfilm of collodion to prevent adhesion of the paraffin to the gelatin. These microfilms were made from "Peerless Microfilm Solution" stretched on a metallic round frame at the surface of water (25°C.). It was found that, by working in a cold room at 0°C. for placing the sections in contact with the emulsion, the prevention of the adhesion of the paraffin to the gelatin was more complete altho the material was subsequently kept at room temperature.

Various photographic materials were assayed, including X-ray film, microfilm film, fast panchromatic film, Lipmann "fine grain" film, orthochromatic film and various types of lantern slide plates. The Lipmann emulsion was not sensitive enough to react. Similarly the microfile film showed only weak reactions. In the case of the orthochromatic film, the roughness of the surface of the film complicated the interpretation of the pictures. Satisfactory results were

obtained with the other types. Medium lantern slide plates (Eastman Kodak) were selected for routine work since they were more conveniently handled than the films.

The slides bearing the sections, prepared as described above, were placed on the sensitive face of medium lantern slide plates in the dark room or in a dark cold room. Pressure was exerted to keep close contact between the sections and the plates. For that purpose various devices may be used. Enclosure of the sections and the plates in X-ray exposure boxes were found convenient. The length of time during which the contact between plate and section must be main-



A and B—Thyroid treated by routine histological technic (Masson's trichrome stain) as compared with its autograph. The parathyroid in upper left corner and the muscles with connective tissue in upper right corner do not react on the photographic plate, contrary to thyroid tissue.

C and D—Thyroid treated by freezing drying technic and corresponding autograph. A small portion of parathyroid is visible in upper right corner.

tained varied according to the intensity of the radioactive iodine per unit of thyroid tissue. With I^{130} the slides were usually kept in contact for a week when the radioactivity of the tissue was practically exhausted. The intensities used with I^{130} were usually of the order of 0.5 to 10 microcurie per gram of thyroid tissue at the time when the sections were placed on the plate. The autographs were not intense. Much clearer autographs were obtained using similar intensities with

the long life isotope I^{131}, with which the effect may be extended for long periods of time. We found, however, that with the available intensities, not much benefit was derived from prolonging the exposures for over three weeks.

At the end of the exposure, the development and the fixation of the plate were carried out as usual (Eastman universal developer powder and acid hyposulphite fixative). The sections were then stained by either Masson's trichrome for routine sections or by hematoxylin-eosin after absolute alcohol treatment for the freezing-drying sections[2]. Comparisons of the stained slides and the "autographs" on the photographic plate were done with the help of a binocular dissecting microscope or on photographic enlargements.

Since detailed histological results are published elsewhere, it is sufficient to state that definite autographic pictures were found only in the thyroid gland, and that in this organ most of the iodine was apparently located in the colloid and predominately in the acidophilic one. Small doses of iodine, such as one microgram, injected in a rat produced clear-cut autographs indicating localization of the radioactive iodine in the colloid. In this condition, chemical analysis has shown the presence of large quantities of organically bound iodine.

On the other hand, soon after injection of large doses of iodine, such as one milligram (to a rat), when the iodide ion was present as such in the gland (Leblond, 1941) the autographs showed diffuse pictures, even after freezing-drying fixation. It is possible that the iodide was displaced during paraffin embedding. Another explanation is that the picture may be truly representative of the diffuse distribution of the iodide thruout the epithelium and colloid.

The technics used in this work were devised with the help of John F. Bonner, Jr., to whom we wish to express our appreciation. We are also indebted to Dr. Stafford L. Warren and W. F. Bale for assistance.

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[2]Thyroid sections obtained by the freezing drying technic were characterized by a homogeneous colloid which stained basophilic; no peripheral vacuoles were found; only an occasional round vacuole could be seen in the centre of a colloid mass, altho many animals showed none.

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THE SURFACE STAINING OF EMBEDDED TISSUES[1]

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ABSTRACT.—**The staining procedure is based on the theory that the freshly cut surface of embedded material will absorb stain only in the exposed tissue elements, provided that the embedding compound itself will not absorb the staining fluid. Concentrated stains are used for short intervals to insure minimum penetration. For paraffin embedded materials: (1) Cut block, preferably on microtome, to the desired tissue surface. (2) Rinse in absolute alcohol. (3) Float face down in stain. (Ripe, concentrated alum hematoxylin —Galigher's formula recommended—will stain in 10 to 15 minutes. Heidenhain's iron hematoxylin works exceptionally well in some cases.) Mordant 20% alum 5 to 10 minutes, briefly rinse, and stain comparable 5 to 10 minutes in 1 to 1.5% hematoxylin. (4) Allow to become blue in tap water (for hematoxylin stains). (5) Counterstain if desired. (6) Dehydrate in absolute alcohol for not more than 10 minutes. (7) Dry for 15 to 20 minutes. (8) Trim block to 2-3 mm. and mount between two cover glasses by use of microflame. Attach mount to slide with balsam. For celloidin embedded materials: (1) Dehydrate block with 90% alcohol, phenol-toluene, finally pure toluene. (2) Rinse cut surface with 90% alcohol, then apply stain. (3) Wash, after hematoxylin stains, counterstain if desired. (4) Dehydrate surface, 90% alcohol, phenol toluene, pure toluene, and mount in medium dissolved in toluene.**

Possible applications of surface staining technic are suggested and illustrated.

INTRODUCTION

The details of the following technic resulted from an effort to stain a sagittally cut salamander brain in such manner that only the cut surface would be stained. The purpose was to emphasize the relation of sagittal elements to the rest of the cavities and structures of the brain. This was accomplished and utilized in a previous publication (Copeland, 1943, Fig. 4). Developing the surface stain technic led to refinements not in immediate line with requirements. At the risk of accusation of "technic for technic's sake", I present the total of those developments with notes as to possible applications.

It has become general practice to soften refractory, paraffin-

[1]A portion of the material described in this article was presented as a demonstration at the A. A. A. S Meeting, Dallas, Texas, December, 1941. (Copeland, 1941).

embedded tissues by exposing the tissue surface and soaking the block in water overnight or longer. Such tissues imbibe water to the extent that they often bulge noticeably from the cut surface. Baker (1942) investigated a number of solvents and recommends 9 parts of 60% alcohol and 1 part of glycerin as a most effective softening fluid.

Penetration is comparatively slow. It probably takes place by way of the coagulated plasma and cellular elements because no penetration occurs unless the tissue is exposed. Many workers have taken advantage of this type of diffusion to stain serial paraffin sections by floating the ribbons on an aqueous stain (Smith, 1899; Diettrich, 1915; McFarland, 1922; Walsem, 1931; Tahmisian and Slifer, 1942). Tissues so treated become stained in their entirety.

The theory of "surface staining" outlined in this article requires that the tissue be embedded in material that does not absorb the stain. Only the cut, exposed tissue elements take the stain. A short exposure to a concentrated solution results in a stained "section" comparable to the thinnest of microtome sections.

Paraffin Embedded Material

Technic. The block of paraffin is cut, preferably on a microtome, to expose the tissue-surface that is to be studied. After rinsing for several seconds in absolute alcohol to insure a receptive surface, the block is floated face down in the stain.

Heidenhain's iron hematoxylin often gives excellent results (Fig. 2). To prevent excessive penetration of fluids and consequent swelling of tissues, it is found best to mordant for 5 to 10 minutes in 20% iron alum, rinse several seconds in distilled water and then to stain in ripe 1.0 to 1.5% hematoxylin for a period equal to that of mordanting. Alum hematoxylin is a more reliable and consistent stain than Heidenhain's. Galigher's formula for this stain was used, since it permits an increase in the concentration of hematoxylin. A mixture that is ripe enough and of sufficient concentration to stain mounted sections in 3 minutes, should give a good surface stain in 10 to 15 minutes (Fig. 3). Ehrlich's hematoxylin is advisable if it is difficult to get good nucleo-cytoplasmic differentiation with the preceding methods, or if a deeper penetration of stain into the block is desired. For best results, the concentration of hematoxylin should be doubled or tripled.

After hematoxylin staining, the block is washed in running tap water for a period equivalent to the staining interval. Counterstaining with stains such as eosin or orange G is done best in 95% alcohol. This at the same time initiates dehydration which is then completed in absolute alcohol. Longer than 10 minutes in absolute

alcohol is not advisable, as the alcohol tends to dissolve out elements in the paraffin, leaving it white and brittle. The block is gently blotted and permitted to dry 15 minutes or longer.

Finally the block is trimmed to 2 or 3 mm. in thickness and mounted. A cover glass (cut to size by a diamond) with edges projecting beyond the block is sealed to the stained surface by means of heat. Mounting media are not feasible, because their solvents dissolve the paraffin and eventually precipitate it again as obscuring crystals. For best results the trimmed block is placed face up on a chilled copper plate. Then a microflame is pointed perpendicularly at a corner of the plate, and the plate approached until faint moisture condensation appears. The so-determined distance of flame from plate is usually the best one to use in annealing on the cover glass, starting at one edge of the cover glass and sweeping across as rapidly

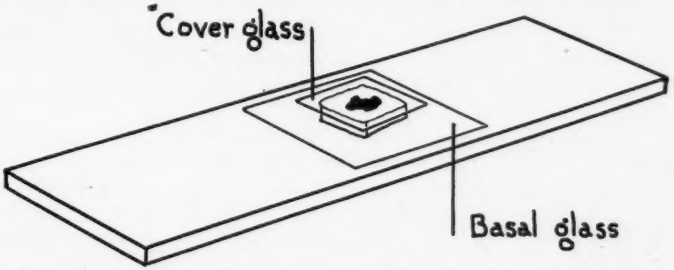


FIG. 1. A sketch illustrating the method of mounting the paraffin block. The cover glass, then the basal glass are sealed to the block by a microflame. Finally the basal glass is fastened to the slide with mounting medium.

as the film of melted paraffin appears under the cover glass. Careful use of the flame will insure the cover glass being so closely sealed to the tissue-surface that no crystals will intervene.

A basal glass (Fig. 1) is attached by the same method and the complete unit is sealed to a slide with mounting media. If oil immersion is to be used it is necessary to have the cover glass and tissue-surface parallel to the slide. If the basal glass is large enough, quite often it "balances" and levels itself as the paraffin is melted. Otherwise, glass rods are needed between cover glass and basal glass to obtain parallel surfaces.

As mentioned in the introduction, paraffin serial sections may be stained while in ribbon form. Mounted paraffin sections that are to be stained thru their complete thickness should be soaked in absolute alcohol for a minute or two, then put directly in the stain. The resulting, strong diffusion gradient aids in penetration of the

Plate 1

Explanation of Figures

2. Amblystoma intestine, paraffin embedded. Stained with Heidenhain's iron hematoxylin, mordanted 5 minutes, stained 5 minutes. No counterstain. Note the cytoplasmic texture and sharpness of cellular details. ×480.

3. Amblystoma intestine, paraffin embedded. Stained with alum hematoxylin for 10 minutes, counterstained with orange G. The "surface" character of the stain is indicated by the fact that of the crowded nuclei only those at the cut surface are stained. ×320.

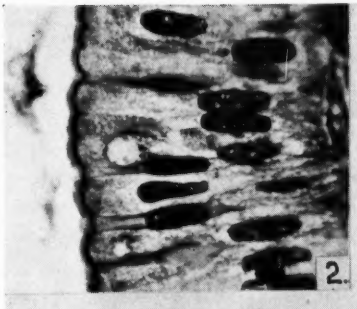
4. Amblystoma intestine, paraffin embedded. Stained with alum hematoxylin for 15 minutes, counterstained with orange G. Surface of paraffin is in focus. Note that the base and nucleus of the goblet cell are in focus, while the deeper lying body is not in focus. ×700.

5. Same as Figure 3 but with a deeper focus to show that stain (orange G) has traveled down the goblet cell. No other elements at this level received stain.

6. Triturus brain, paraffin embedded. Cut with microtome down to sagittal section. Surface stained with alum hematoxylin. Photographed in 70% alcohol after removal of paraffin. Note pituitary, infundibulum, III and IV ventricles, and nuclear layers. ×6.

7. Amblystoma intestine, celloidin embedded. Stained with Ehrlich's acid hematoxylin for 15 minutes, no counterstain. Note globules in the unopened goblet cells. These are slightly below the celloidin, cut surface. ×550.

8. Recently hatched squid, celloidin embedded. Stained with alum hematoxylin for 10 minutes. Counterstained overnight in a mixture of orange G and eosin Y diluted in 80% alcohol. The result is a "frontal section" with a three-dimensional background. ×4.5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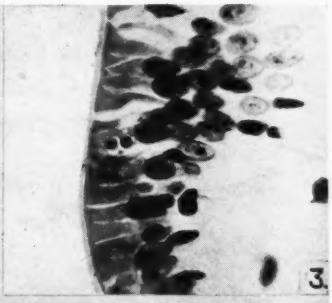
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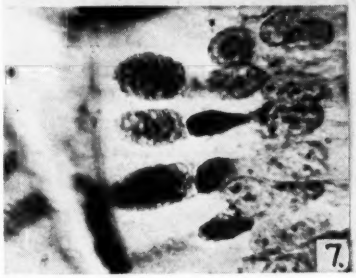
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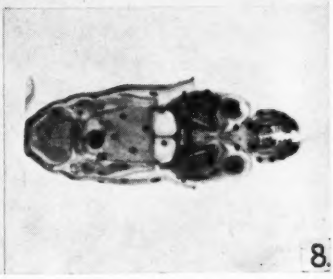
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stain. Attempts were made to "surface stain" mounted sections of 10 to 20 μ. The results were not as uniform as those obtained with intact block-surfaces. The passage of the ribbon over the knife edge no doubt disrupts the crystalline structure of the paraffin, and the heat used in spreading the sections may cause small areas to be covered with traces of paraffin, thus causing uneven penetration of stain.

Discussion of applications. The technic of "surface staining" is not applicable to the usual serial sections, but nevertheless may be of considerable use in some cytological problems. Tissues left in the paraffin block and surface-stained to a depth of 3 or 4 μ do not suffer the compacting distortion found in sections that are cut to this thickness. Nitrocellulose embedding would prevent distortion, but nitrocellulose sections can be cut to an equivalent thickness only by the finest technic.

The modified Heidenhain technic gives minimum penetration. Note in Figure 3 that altho the nuclei are crowded there is no sign of overlapping or superimposed nuclei. Furthermore, the cytoplasmic bodies of the cells are distinct, since only their surfaces are stained. In this particular case the degree of cytoplasmic shrinkage due to fixation and embedding can be accurately gauged.

Longer staining periods give greater penetration, being most rapid along the tissue elements which extend inward from the cut surface. This often gives an interesting microscopic, three dimensional picture. To illustrate: in Figure 4 the cut surface is in focus. In Figure 5 the focus is below the surface showing the degree of penetration of the stain into a goblet cell whose nuclear end is at the level of the cut surface. Other nuclei and cytoplasmic structures at this level which have no connections with the surface do not stain. This phenomenon may be of aid in studying tissue groups or continuities. Prolonged staining will affect all tissue elements to a considerable depth. Such preparations are best stained with a self-differentiating stain such as Ehrlich's acid hematoxylin. The deeper-stained tissues are limited in usefulness to the extent of their visibility thru the paraffin.

A block of paraffin 2 to 3 mm. in thickness easily transmits light and at the same time diffuses it so that the stained tissues have a smooth, brilliant appearance. If the embedded material contains no pigment clumps, oil immersion may be readily used. This type of lighting is of some advantage in photomicrography. All that is required is a strong light-source and the substage condenser raised to a position which gives the most concentrated light. Diffusion screens,

diaphragm focusing, source focusing, etc. are not necessary, because the diffusing action of the paraffin would negate such arrangements.

Material containing water-soluble components such as glycogen may be stained by this technic. Only cursory investigations were made of this aspect, so no critical recommendations are made here.

This technic is also of aid in the study of friable materials such as heavily yolked eggs.

The technic can be recommended for use where microtomes are not available, such as in field work or in small schools. Satisfactory results can be obtained by cutting and trimming the block for surface-staining by using an old-fashioned sectioning razor or a one-edged safety razor blade.

This method may also be used in preparing anatomical and embryological demonstrations. For example, an embedded brain or embryo is cut with a microtome down to the mid-sagittal region and surface stained. Then, removal of the paraffin by solvents and hydrating down to 70% alcohol reveals a "sagittal section" (mentioned in introduction) with a three dimensional background (Fig. 6). A cover glass should be sealed to the stained surface before removal of the paraffin. Otherwise, the tissue absorbs the solvent and as a result swells and buckles at the exposed surface. Even with this precaution, tissues composed of alternate layers may become distorted due to differential shrinkage. The whole process must be done slowly and with due regard for convection currents in the alcohols.

Celloidin-Embedded Material

Technic. It is more difficult to obtain a surface stain with celloidin material because water-soluble stains penetrate readily and deeply into celloidin. In an attempt to make the celloidin less receptive to entrance of aqueous stains, it was impregnated with various water-immiscible fluids. Of the common solvents, toluene was found best.

The tissue-blocks are sectioned to a desired thickness (*ca.* 2 mm.) and stored in 80% alcohol until needed. At that time they are dehydrated in 90% alcohol (a higher alcohol will soften the celloidin) and transferred to equal parts of phenol and toluene until clear, then to pure toluene. To prepare the surface for staining, the block is picked up on a section-lifter and rinsed briefly with 90% alcohol from a medicine dropper. The stain is then quickly applied to the tissue-area by means of another medicine dropper. Staining periods are usually shorter than those used for paraffin-embedded material. Alum hematoxylin and Ehrlich's acid hematoxylin work best.

Removal of the staining fluid shows the celloidin surface whitened

by the mixing of toluene and water. After washing in tap water, the blocks are placed in 90% alcohol until they again become clear. Counterstaining is hard to control, but can be done at this stage. Lastly, the block is dehydrated for mounting by use of phenol-toluene, followed by pure toluene. If the mounting medium has xylene as its solvent, then xylene is used in place of toluene in the last two steps.

Discussion of applications. The above technic does not furnish surface stains comparable to those obtained on paraffin-embedded material. Penetration is usually deeper, and the degree of staining is harder to control.

This method may be of use in study of formed objects such as globules of mucin in goblet cells (Fig. 7). In this particular case the cells can be seen in their entirety, and need not be traced from serial section to serial section. Also there are the well-recognized advantages of no displacement by the knife blade, and no distortion by heat of embedding such as are found in paraffin ribbons.

Celloidin surface staining is posibly best adapted to the preparation of material for classroom instruction. Gastrulas, embryos, etc. can be sliced in two, each half surface stained, and mounted (Fig. 8). This gives a "sagittal section" with a three dimensional background such as is mentioned above in the paraffin technic. Moreover, in this case the celloidin need not be removed and has the advantage of holding loose internal tissues in position.

If the deeper-lying tissues have no pigment, they may become transparent and almost invisible in the mounted preparation. In that event, the tissues are given a counterstain by soaking the block overnight in a weak concentration of counterstain (in 80% alcohol). Concentration is determined by trial.

Summary

1. The "surface staining" of a cut surface of an embedded tissue requires that the embedding compound does not absorb the staining fluid. Consequently only the exposed, surface elements of the tissue will receive stain.

2. The shorter the staining interval, the shallower is the resulting stain and the thinner the "section." Shorter staining intervals are obtained by increasing the concentration of the stains.

3. The technics of surface staining of paraffin-embedded tissues with Heidenhain's iron hematoxylin, alum hematoxylin, Ehrlich's acid hematoxylin, and counterstains are outlined. A method is given for the mounting of the final paraffin block without the aid of mounting medium.

4. The principles of surface staining of celloidin-embedded materials are also outlined. The chief difficulty encountered in this technic is to make the celloidin suitably resistant to penetration of aqueous stains.

5. Some of the possible applications and advantages inherent in surface staining are enumerated.

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HANDLING SMALL OBJECTS IN BULK FOR CYTOLOGICAL PREPARATIONS

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Metz's method[1] of using the abdominal portion of the inner membrane of *Drosophila* pupae, at about the middle of the pupal stage, as transfer-sacs for handling small objects during fixation, washing, dehydration and embedding of small objects is both ingenious and useful. The principal handicaps of the technic lie in: the required

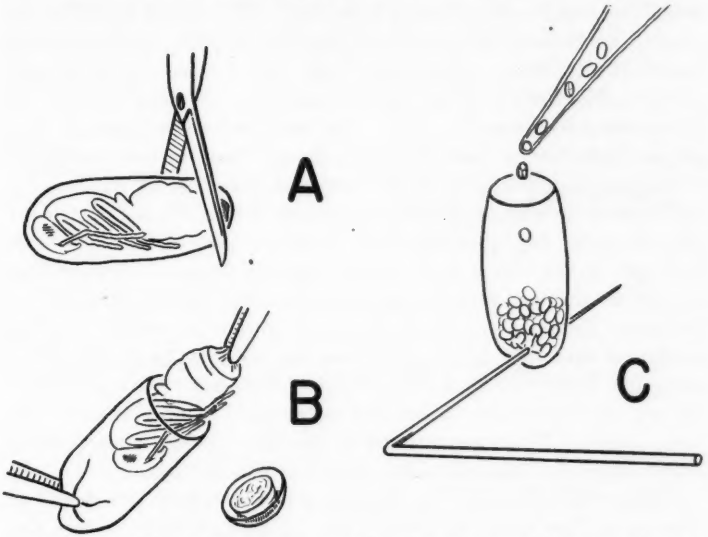

FIGURE 1. Method of preparing transfer-sacs from cocoons of camponotine and ponerine ants. A, location of cut; B, removal of pupa,—the caudal tip of the cocoon containing the meconium, etc., and the tip of the pupal abdomen is at the lower right; C, method of holding the cocoon erect while filling it.

maintenance of *Drosophila* stocks, or laborious preparation of a large number of the sacs in anticipation of future needs; the rather difficult preparation of the sac itself; and the relatively limited sizes of pupae available among easily bred flies. The same objections likewise can be levelled against Cooper's method[2] of preparing transfer-

[1]Metz, C. W. 1921. A simple method for handling small objects in making microscopic preparations. Anat. Rec., **21**, 373–4.

[2]Cooper, K. W. 1939. The nuclear cytology of the grass mite, *Pediculopsis graminum* (Reut) . . . Chromosoma **1**, 51–103.

sacs from *larval* integuments of *Drosphila*, after evisceration, by hardening them in 95% alcohol while stretched over the tips of blunt needles.

The cocoons of ponerine and camponotine ants[3] may be put to similar use, and are here recommended as perhaps the most satisfactory natural envelopes for handling microscopic materials such as large protozoa, arthropod eggs, minute dissections, etc. Such ant pupae may easily be collected in large numbers from ant nests under stones or bark, and preserved in 70% alcohol until required. When it is desired to transfer minute objects in bulk, a sufficient number of ant pupae are run from the alcohol to a stender dish of the fluid from which the minute objects are to be moved. The caudal tip of the ant cocoon, containing the cast larval skin, peritrophic membranes and meconial mass which collectively form a dark brown or black spot easily visible thru the wall of the cocoon, is cut off along with the tip of the pupal abdomen (Fig. 1,A). The head end of the cocoon is then gripped with forceps, and the pupa removed thru the cut caudal end of the case (Fig. 1,B). A small sharpened wire bent at right angles (still better, a bent entomological "minuten-Nadel") is then thrust thru the intact cephalad end of the cocoon. The cocoon then stands vertically in the dish of fluid, and the minute objects to be carried in the sac are dropped into the opening with either pipette (Fig. 1,C) or forceps. The open end of the cocoon may then be closed by either stuffing it shut with guts pulled from the abdomen of the discarded ant pupa, or twisted shut with forceps. Thereafter the cocoon containing the object may be handled in the same manner as any other large object. Following paraffin or paraffin-celloidin embedding it cuts beautifully, forming neatly circumscribed sections.

Unlike the permeable but continuous inner pupal membrane of *Drosophila*, the spun silk pupal cases of ants resemble a very fine netting. The microscopic meshes allow a more rapid fluid exchange between the contents of the transfer-sac and the outer medium. A further virtue of using ant cocoons is that the size range is large; numerous species having cocoons ranging from approximately 1.5× 0.5 mm. to somewhat more than 8×3.2 mm. being easily obtainable in most sections of the United States. Those of the species of *Lasius* (mostly pale yellow ants living under stones and logs), which are about 2.5×0.4 mm., have been found especially serviceable. Lastly, the method is technically much simpler that than of using *Drosophila* inner pupal membranes.

[3]Wheeler, W. M. 1926. Ants, their structure, development and behavior. Columbia Univ. Press, N. Y.

A USEFUL ACCESSORY TO THE ZEISS MECHANICAL STAGE FOR OIL IMMERSION MICROSCOPY

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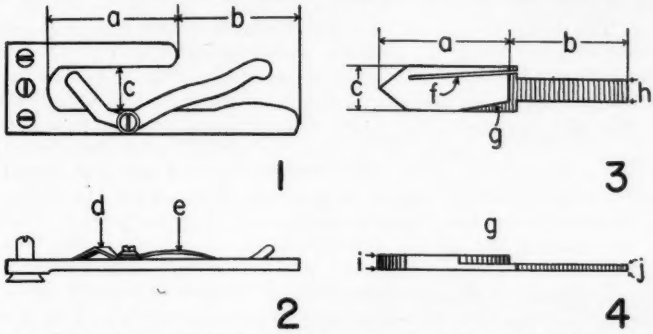
Oil immersion of the condenser as well as the objective is a prerequisite to obtaining full use of the high apertures of homogeneous oil immersion objectives. Unfortunately the design of the mechanical stage (Zeiss Rectangular Mechanical Stage G) supplied on many or indeed most of the Zeiss research stands in service in this country discourages the use of oil between condenser and slide. With this stage the microscope slide rests on the stage plate itself, being held in place by two slide-holders which move it laterally over the surface of the stage. When full lateral excursion of the mechanical stage is



FIGURES 1 and 2, top and lateral views respectively of the right Zeiss slide-holder. FIGURES 3 and 4, same of the adapter. a, b and c linear dimensions which are equal in Fig. 1 and 3; d, phosphor-bronze clip; e, clip of slide-holder; f, slot in adapter; g, excised wedge; h, breadth of slide-carrying arm of adapter; i, thickness = 3.5 mm.; j, thickness = 1.1 mm.

employed, the microscope slide draws oil between itself and the vulcanite surface of the stage plate. The results are exasperating, for not only is there a considerable loss of oil from the condenser itself and a gumming of the stage with immersion oil, but the reverse lateral movement will now frequently introduce air bubbles between the condenser top lens and the slide. To avoid these difficulties the recent Zeiss stages are provided with clips on the long arms of the slide-holders (Fig. 1; 2, e). By moving the slide-holders closer together and placing the microscope slide *on* the arms themselves and *under* the clips, the above defects are remedied. But this solution of the problem awakens new difficulties, for owing to the absence of fixed

stops on the slide-holders, the slides can rarely be placed on the holders in the same position on consecutive trials. The result is that the mechanical stage vernier readings are at best only very approximate, and the virtues of the graduated stage are largely lost for high power microscopy.

The device here described is extremely simple to make, and completely obviates the difficulties inherent in the Zeiss mechanical stage itself. It consists of two machined brass slips which snugly fit the space between the long and short arms of the slide-holders on each side of the stage. These slips or adapters in effect form raised platforms above the stage plate upon which the slide normally rests. The adapter figured (Fig. 3, 4) is for insertion in the right slide-holder (Fig. 1, 2); the design for the left differs merely in being its mirror-image. Actual dimensions of the adapter slip, except those of thickness, are not given, for there is some variation among slide-holders; other dimensions—such as the width h, Fig. 3—may be freely varied without altering the performance of the adapter. It is important only to note the following facts.

(1) The points of juncture between segments a and b, Fig. 1 and 3, should coincide when the adapter is slid into slot c (Fig. 1) of the slide-holder. (2) By slotting the adapter slip (Fig. 3, f), the inner face acts as a spring holding it firmly in place in the slide-holder. (3) The best thickness (Fig. 4, j) for the slide-carrying limb (segment b, Fig. 3) of the adapter is 1.1 mm. (about 0.5 mm. less than the thickness of the arms of the slide-holders, and 2.4 mm. less than the thick a-segment of the adapter [Fig. 4, i]). Thus, when the adapters are placed in the slots of the slide-holders, the microscope slide exactly fits the holders just as tho it were resting on the stage plate itself, while in fact it is lifted sufficiently above the stage plate so that full lateral motion of the mechanical stage may be obtained without wetting the stage with the immersion oil on the lower face of the slide. (4) A wedge is cut from the side of the adapter contacting the long lateral arm of the slide-holder (Fig. 3, g; 4, g) so that the clip (Fig. 1; 2, e) of the slide-holder may be used to secure the slide in place on the adapter. (5) There remains the placing of a short but strong phosphor-bronze or spring-steel clip under the base of the slide-holder clip (Fig. 1; 2, d). This accessory clip presses the thick body of the adapter firmly to the stage and prevents dislocation of the adapter while it is carrying the microscope slide over the central well of the stage plate.

The advantages of this device are three: the deficiencies of the slide-holder design for oil immersion microscopy of the Zeiss mechanical stage G are completely remedied; the calibration, precision and readings of the mechanical stage remain unaltered; and lastly no machining of the hardened steel slide-holders is necessary.

A CORROSIVE SUBLIMATE FIXING SOLUTION FOR YOLK-LADEN AMPHIBIAN EGGS

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Up to the present, no really satisfactory reagent has been described for the fixation of yolk-laden amphibian eggs which are being preserved for sectioning. The common laboratory fixatives render the eggs very hard and brittle with the result that they crack badly in sectioning. The dioxane-paraffin method, described by Puckett,[1] is a very satisfactory one but it was felt that there must be some common laboratory fixative which could be used to preserve these eggs without rendering them too brittle for satisfactory sectioning.

With this problem in mind, a great deal of time has been spent in this laboratory testing a large number of existing and newly devised fixatives in an attempt to find a really satisfactory fixative for this type of material. Other steps in the paraffin technic such as dehydration, clearing and paraffin infiltration have been carefully investigated and we feel that the technic to be described is a really satisfactory one. Excellent sections can be obtained by sectioning the material shortly after fixation, but equally good results have been obtained from eggs stored for a year after fixation. The procedure is as follows:

1. Fix small masses of living eggs, including the adherent jelly masses, for 48 hours in fairly large volumes of the following modification of Worcester's fluid:

Saturated aqueous $HgCl_2$	90 ml.
Formalin (full strength)	8 ml.
Glacial acetic acid	2 ml.

(After fixation the eggs may be stored in 5% formalin for long periods.) Formalin harden less than 70%

2. Wash in dishes of water holding 150–200 ml.; make six changes over a period of one hour in order to remove fixative.

3. Place a small cluster of eggs with the adherent jelly in a test tube one-third filled with water. Shake vigorously. Repeat this washing several times, shaking less vigorously with each change of water. This treatment will remove the greater part of the jelly.

[1]Puckett, William O. 1937. The dioxan-paraffin method for sectioning frog eggs. Stain Techn., 12, 97–8.

4. Wash the almost jelly-free eggs in a partially filled test tube of 1% NaOCl solution. Shake gently for several minutes. Careful treatment at this stage will result in the removal of the remaining jelly and in a dissolution of the vitelline membrane.

5. Wash in six to ten changes of water over a period of several hours in order to remove all traces of the hypochlorite solution.

6. Dehydrate thru 35%, 50%, and 60% alcohol. Allow two hours for each step.

7. Treat with 70% iodized alcohol for 24 to 48 hours in order to remove all traces of the corrosive sublimate. Wash for one hour in 80% alcohol.

8. Dehydrate thru 95% alcohol (one hour) and 100% alcohol (two hours, changing once).

9. Clear in xylene for 30 minutes, change xylene and continue clearing for an additional hour.

10. Transfer eggs to a mixture of one-third xylene and two-thirds soft (48°) paraffin kept at room temperature. The eggs can be kept in this mixture for several hours without harm.

11. Place eggs in this xylene-paraffin mixture in oven at 58°C. until the mixture is completely liquified. Replace this mixture with 52° paraffin for one hour. Transfer to 55° paraffin for three hours, or longer. Embed in 55° paraffin.

12. Section eggs at 5–15 μ, with the microtome knife set to give a rake angle of 70–80°.

13. Mount and stain sections in the usual manner. We find the best stain to be Delafield's hematoxylin with an eosin-orange-G mixture as a counterstain. This counterstain is made in the following manner:

1% solution of eosin in 95% alcohol	10 ml.
Saturated solution of orange G in 95% alcohol	5 ml.
95% alcohol	45 ml.

DIOXANE AS AN AID IN STAINING INSECT CUTICLE

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In working with three genera of Ephemerida (mayflies), it was found necessary to devise a new technic to stain serial sections of insects that had been fixed and preserved in a 70% alcohol solution in a jar with the level of the preservative just reaching the top of the mass of insects. Since alcohol at best is a poor fixative, this low percentage of alcohol and insufficient amount of fluid added to the difficulty.

With the routine hematoxylin-eosin technic, very poor and variable results were attained with sections cut at 10–12 μ. Of a number of other technics used, the following gave the best results:

1—Xylene, 2–3 minutes
2—Xylene, 1 minute
3—100% dioxane, 2–3 minutes
4—100% dioxane, 1 minute
5—Methylene blue, saturated solution in 95% dioxane, until the tissues turn blue-green (Nat. Anil. & Chem. Co. Cert. No. NA-48)
6—100% dioxane, wash 5 seconds
7—Erythrosin, saturated solution in 95% dioxane, until the desired contrast is achieved (Nat. Anil. & Chem. Co. Cert. No. NEr-1)
8—100% dioxane, wash 15 seconds
9—100% dioxane, 2–3 minutes
10—Xylene, 2–3 minutes
11—Xylene, 1 minute
12—Mount in gum damar under a coverslip

A more complete differentiation between the three layers of cuticle will result if the methylene blue solution is allowed to stand in a stoppered bottle for a week or more before use.

As a result of this technic, the following details were noted. The nuclei of the cells in connective, epithelial, fatty, and glandular tissues were stained a light blue and were visible only under the 4 mm. and oil immersion lenses. The cytoplasm of these tissues, as well as muscle fibers, nerve cord, and the hypodermal layer stained a red-pink. The epicuticle showed as a thin black line, the exocuticle

graded from yellow to olive-green, and the endocuticle was seen as a blue translucent layer.

It is easy to keep stock solutions of dioxane free of water by adding a small amount of anhydrous CaO to the bottle. This method may also be used to dry solutions of dioxane after they become contaminated with stain solution. Since most of the dyes are insoluble in pure dioxane, as stated by Mossman, they will precipitate out while the drying is in progress.[1]

It is to be noted that solutions No. 3, 4, 8, and 9 should be changed frequently, for they soon become contaminated with water and may ruin the preparations. If any destaining is found to be necessary, it can easily be accomplished by dipping the slides for a few seconds in a 95% solution of dioxane. This time is quite sufficient for the most overstained tissues and the destaining may be promptly halted by immersing the slide in a solution of pure dioxane. Dioxane evaporates very rapidly and precautions must be taken to prevent the sections from drying during inspection. This technic can also be used in regular histological procedure, as pointed out by Mossman, but it is best to use hematoxylin instead of methylene blue as a nuclear stain.

The dioxane technic works well in the case of tissues that have been allowed to remain in fixatives for long periods of time and which fail to stain with the routine technics. In working with *Squalus* embryos that had been fixed and preserved in Bouin's fluid for years, Heifetz found that sections failed to stain with the routine technics. A saturated solution of crystal violet in 95% dioxane used as in the above technic produced well stained sections.

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[1]Mossman, H. W. 1937. The dioxane Technic. Stain Techn., **12**, 147–56.

A CONTROLLABLE SILVER STAIN FOR NERVE FIBERS AND NERVE ENDINGS

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ABSTRACT.—**A paraffin section method is described with a yellow-brown-black color range comparable to that of Ranson's pyridine silver block stain. After impregnation with activated protargol and reduction with a fine grain photographic developer, silver nitrate impregnation and reduction are repeated as often as necessary. The procedure is as follows:**

Place hydrated sections of tissue fixed in chloral hydrate (25 g. in 100 ml. of 50% alcohol) in 1% aqueous protargol (Winthrop Chemical Co.) containing 5–6 g. metallic copper for 12–24 hours. After rinsing in 2 changes of distilled water, reduce 5 to 10 minutes in: Elon (Eastman Kodak Co.) 0.2 g., Na_2SO_3, dessicated, 10 g., hydroquinone 0.5 g., sodium borate powder 0.1 g., distilled water 100 ml. Wash thoroly in 4 or 5 changes of distilled water and place in 1% aqueous $AgNO_3$ for 10–20 minutes at 28°–50° C. Rinse in 2 or 3 changes of distilled water and reduce in the elon-hydroquinone solution. After thoroly washing in 4 or 5 changes of distilled water, examine under microscope.

If too pale, treat again in silver nitrate for 10–20 minutes, rinse, reduce 5–10 minutes and wash thoroly until nerve fibers show distinct microscopic differentiation, then dehydrate, clear and mount.

INTRODUCTION

By modifying Cajal's block method, Ranson[1] was able to distinguish unmyelinated nerve fibers by color as well as by size. Nonidez's modification[2] of the same method, when applied to the peripheral autonomic nervous system, stains all elements intensely except postganglionic sympathetics. As is the case with most block stains, these methods often show the periphery overstained, while the center is understained; only the intermediate zone is useful. The obvious solution of this problem is a section method of staining such

[1]Ranson, S. W., and Billingsley, P. R. 1918. The superior cervical ganglion and the cervical portion of the sympathetic trunk. J. Comp. Neurol., **29**, 313–58.

[2]Nonidez, J. F. 1939. Studies on the innervation of the heart. Amer. J. Anat., **65**, 361–413.

as Bodian[3] has devised. In the section methods with which we are familiar, the differential staining of fibers is lost by toning with gold.

In attempting to devise a section method that would provide a color range comparable to that of Ranson's pyridine silver, and at the same time give uniform staining of the tissue, we have used fine grain photographic developers on sections impregnated with activated protargol and $AgNO_3$.

Method

The principle of this method is that the silver is deposited on the tissue step by step under microscopic control. Silver impregnation and reduction are repeated as often as is necessary to obtain the desired result.

1) Fix tissue in chloral hydrate (25 g. in 100 ml. of 50% alcohol)[4].

2) Remove paraffin from mounted sections and hydrate in the usual manner.

3) Place sections in 1% protargol (Winthrop Chemical Co.) containing 5 or 6 g. of metallic copper per 100 ml. for 12 to 24 hours at 37°C., as in Bodian's[3] method.

4) Rinse in two changes of distilled water.

5) Place in the reducer for 5 to 10 minutes, gently agitating during the first minute. To make up the reducer, dissolve the following in the order given in 100 ml. of distilled water at 52°C.:

Elon (Eastman Kodak Co.)	0.2 g.
Na_2SO_3, dessicated	10.0 g.
Hydroquinone	0.5 g.
Sodium borate, powder	0.1 g.

6) Wash thoroly in 4 or 5 changes of distilled water.

7) Place in 1% aqueous silver nitrate for 10 to 20 minutes at room temperature, or at 37° to 50°C.

8) Rinse in 2 or 3 changes of distilled water.

9) Reduce sections in elon-hydroquinone solution, as in step 5.

10) Wash thoroly in 4 or 5 changes of distilled water. Examine under the microscope. If the nerve fibers show distinct differentiation, dehydrate, clear and mount.

11) If the desired result has not been obtained, replace sections in the $AgNO_3$ solution for 10 to 20 minutes, rinse, reduce, wash thoroly and reëxamine. Repeat steps 7, 8, 9 and 10 in this way until the desired result is obtained, keeping in mind that the sections darken during dehydration.

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[3]Bodian, D. 1937. The staining of paraffin sections of nervous tissue with activated protargol. Anat. Rec., 69, 153–62.

[4]See footnote 2.

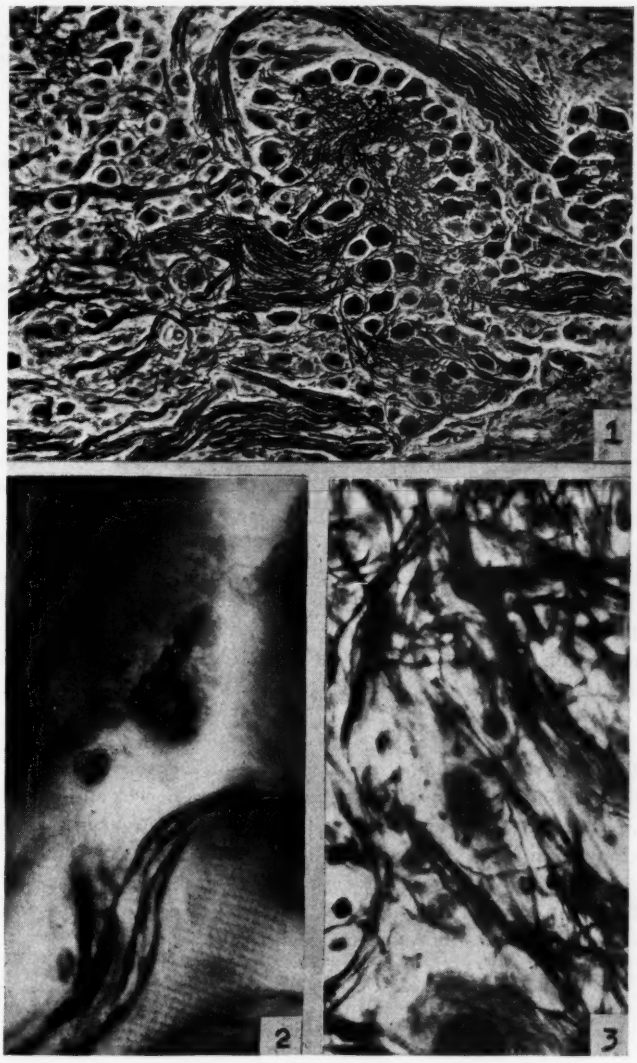

FIGURE 1.

Tissues were fixed in alcohol-chloral-hydrate and sections stained on the slide with the controllable silver method.

1. Sympathetic ganglion of dog; ×150.
2. Nerve endings in striated muscle of dog tongue; ×900.
3. Nerve endings in anterior horn of dog, spinal cord; ×900.

Precautions

Sections must be carefully spread on the slide to ensure adherence and uniform staining. A thin coating of 0.05 to 0.1% celloidin is recommended to prevent the sections from slipping.

The protargol solution should be used only once.

The reducer may be kept for 1 to 2 weeks in the dark at 18 to 20°C. Altho it may be used several times, a fresh supply should be taken from the stock bottle each morning for the day's use.

Altho the $AgNO_3$ solution may be used repeatedly, care must be taken to keep both it and the reducer clear and to change the washing waters frequently to avoid interfering precipitate.

Comment

The number of treatments with the $AgNO_3$ and reducing solutions depends mainly on the fixative used. Our best results were obtained after fixation in chloral-hydrate-alcohol, as advised by Nonidez (footnote 2), but the method is also applicable to tissue that has been fixed in other solutions.

If toning is desired, the sections are placed in 0.2% gold chloride until a grayish color has appeared. They are then rinsed and reduced with elon-hydroquinone which has been diluted 1:20 with distilled water; the reduction should be carefully controlled by microscopic examination. After reduction, the sections are washed, dehydrated, cleared and mounted.

Applications

The innervation of smooth and striated muscle, the plexuses of the gut, autonomic and sensory ganglia, peripheral nerves and neurones, sensory endings in skin, muscle and viscera and sections of the spinal cord and brain have been successfully stained by this method.

I wish to express my appreciation to Dr. Kendrick Hare for his most helpful suggestions and assistance.

DIFFERENTIAL STAINING OF DEGENERATING FASCICLES IN PERIPHERAL NERVES

LUISE H. UNGEWITTER, *Department of Anatomy, Cornell University Medical College, New York City*

Introduction. Degenerating nerves are usually stained by the Marchi method, which selectively impregnates the neutral fat resulting from the degeneration of myelin. Therefore, the method is applicable only to myelinated fibers and to tissue obtained 10 to 30 days

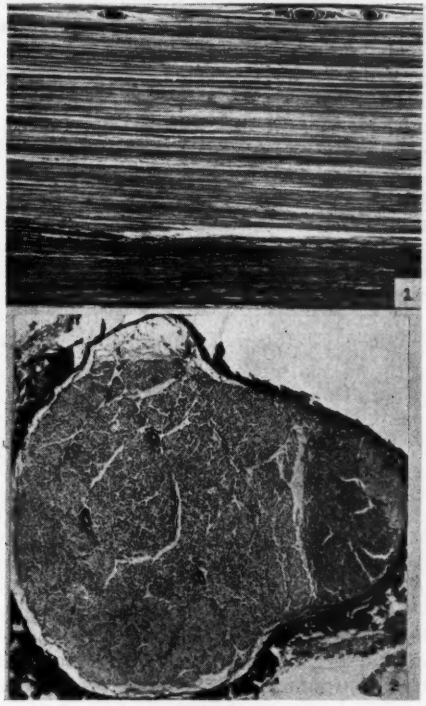

FIGURE 1

Degenerated fibers in the vagosympathetic trunk. Puppy.
1. Longitudinal section; degenerated bundle appears in lower part of figure.
2. Cross section; degenerated bundle to the right.

after injury or operation; furthermore, the method does not consistently yield good preparations. Distortion and fragmentation of axis cylinders can be demonstrated by many silver and general histological methods, but they are limited in application to tissues obtained 4 so

10 days after the lesion has been made. The following method takes advantage of the increased argyrophilia of the Schwann sheath cells and was discovered by a chance toning of a Nonidez[1] preparation of degenerated nerve.

Method. Puppies were autopsied at 2 to 10 weeks after unilateral section of the thoracic ventral roots. The vagosympathetic trunks were removed, fixed in chloral hydrate (25 g. in 100 ml. of 50% alcohol), and stained according to Nonidez[1]. After the paraffin was removed, the sections were toned in a 0.2% aqueous solution of gold chloride containing 6 drops of 10% acetic acid per 100 ml. The toning was carefully controlled by microscopic examination. When the impregnation had reached the desired intensity, the sections were washed, placed in 2% sodium thiosulphate for 2–3 minutes, washed, dehydrated, cleared and mounted.

Variations in impregnation by the silver block method were treated in the following ways:

1) Overstained sections were partly destained in 0.1% potassium ferricyanide[2] or in a diluted (1:10) Weigert's ferricyanide-borax solution, then toned and rinsed in water. If they were still too dark, the bleaching was repeated.

2) Understained sections, before toning, were treated with $AgNO_3$ according to Ungewitter[3]. The initial impregnation with protargol was omitted; the $AgNO_3$ bath and reduction were repeated until the fibers showed the desired intensity of staining.

3) Understained sections, after toning, were treated with a very weak reducing solution of 0.1 g. of Elon in 100 ml. of 2% Na_2SO_3.

Results. In the normal nerve, the sheath of Schwann is stained very faintly or not at all, while the axis cylinders are black. In the degenerated nerve, the hyperplastic sheath of Schwann is a grayish purple and very few axis cylinders are seen. A longitudinal section of the vagosympathetic trunk in which the preganglionic sympathetic fibers are degenerated is shown in Figure 1 and a cross section of this nerve in Figure 2. Unfortunately, the photomicrographs do not show well the striking difference in the staining of the degenerated fascicle; its reddish purple tone permits its prompt identification in the slides under very low magnifications. When the method was applied to sections of spinal cord after the removal of dorsal root ganglia, the differential staining of the degenerating posterior funiculi was very unsatisfactory. It is probable that the method, since it depends upon a Schwann sheath reaction, cannot be successfully applied to the central nervous system.

I wish to express my appreciation to Dr. Kendrick Hare for his most helpful suggestions and assistance.

[1]Nonidez, J. F. 1939. Studies on the innervation of the heart. Amer. J. Anat., **65**, 361–413.

[2]Foley, J. O. 1938. A differential counterstain for toned sections of pyridine silver preparation of peripheral nerves. Anat. Rec., **71**, 133–9.

[3]Ungewitter, Luise H. 1943. A controllable silver stain for nerve fibers and nerve endings. Stain Techn., **18**, 183.

CHLORAZOL BLACK E AS A STAIN FOR ROOT-TIP CHROMOSOMES

JEAN E. CONN, *Commission on Standardization of Biological Stains, Geneva, N. Y.*

ABSTRACT.—**A simple method of staining root-tip chromosomes which gives excellent results in photomicrography is described. The schedule is as follows: run paraffin sections of root tips killed in Bouin's fixative down to water; stain for approximately two hours in 1% aqueous chlorazol black E; wash in water; run up to xylene.**

For photomicrography a black stain, which will appear in the photograph exactly as it appears under the microscope, is desirable.

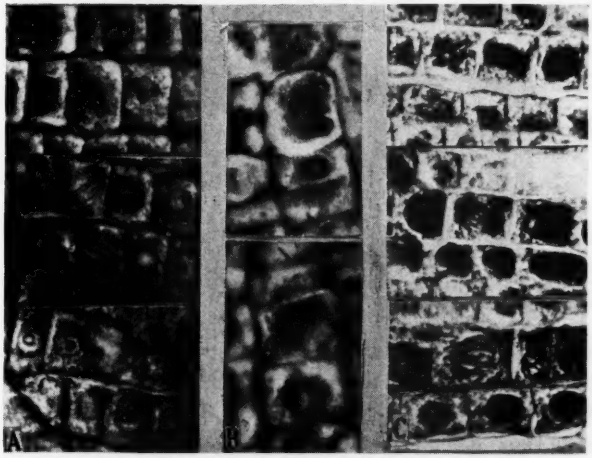

FIG. 1. *Allium* root-tips stained with 0.5% alcoholic chlorazol black E after three different fixatives: A, CRAF; B, Benda's; C, Bouin's. Results rather unsatisfactory.

Hematoxylin, the stain most commonly used for this purpose, requires a long staining schedule with subsequent destaining, and for many procedures the solution must first be ripened for weeks in advance. Recent work with chlorazol black E, an acid dye of the azo group (Colour Index 581), has suggested the possibility that this dye might stain chromosomes in a manner satisfactory for photomicrography, using a much shorter staining schedule than that required for hematoxylin.

Chlorazol black E was first used in England by Cannon (1937) who

did not give much detail concerning its use. This work was followed a few years later by certain American investigations. Nebel (1940) described the use of chlorazol black E as an auxiliary stain in making chromosome counts on plants treated with colchicine in cases where aceto-carmine alone was not satisfactory. He said that it could be used alone or in combination with aceto-carmine. Darrow (1940) proposed a staining schedule using chlorazol black E for sharp differentiation of histological and cytological material. Levine and Morrill (1941) confirmed Darrow's findings, but suggested a slight modification of the staining schedule.

Following these suggestions, an attempt was made to stain the chromosomes of root tips with chlorazol black E. Longitudinal paraffin sections of onion (*Allium*) root tips killed with the following fixatives were used: chrome acetic, CRAF, Benda's, Bouin's, Zirkle's, and Carnoy's.

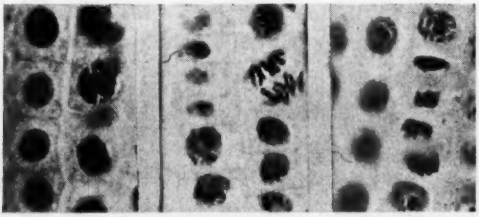

FIG. 2. *Allium* root-tips fixed in Bouin's fluid and stained with 1% aqueous chlorazol black E. Results satisfactory.

The first experiments were carried out using a 1% solution of chlorazol black E in 70% alcohol as recommended by Darrw. According to the schedule she recommended, the paraffin sections were run down to 70% alcohol, transferred to the stain for 5 to 10 minutes, washed in 95% alcohol, and passed thru absolute alcohol and xylene before mounting. The results obtained by this method were not satisfactory because too much dye precipitated on the sections.

Levine and Morrill recommended a 0.5% solution of dye in 70% alcohol and a staining period of 30 minutes. The 0.5% solution proved more satisfactory than the stronger one because in the weaker solution all the dye dissolved, and there was less precipitate on the section. In regard to staining time, the optimum was found to be different for different fixatives. With chrome acetic, Zirkle's, and Carnoy's fluids, the results were unsatisfactory under all conditions; staining following chrome acetic fixation was too faint, and that following treatment with Zirkle's and Carnoy's was too dark. With CRAF, Benda's, and Bouin's fluids, the results were more satis-

factory; with CRAF (Fig. 1a) and Benda's (Fig. 1b) best results were obtained after staining for 30 minutes, with Bouin's (Fig. 1c) a period of 10 to 20 minutes was preferable. This technic gave fairly good general staining, but the chromosomes did not stand out clearly. They were stained best following Bouin's fixative, but there the background was too dark to show the figures well.

In an effort to obtain sharper staining of chromosomes, a 1%

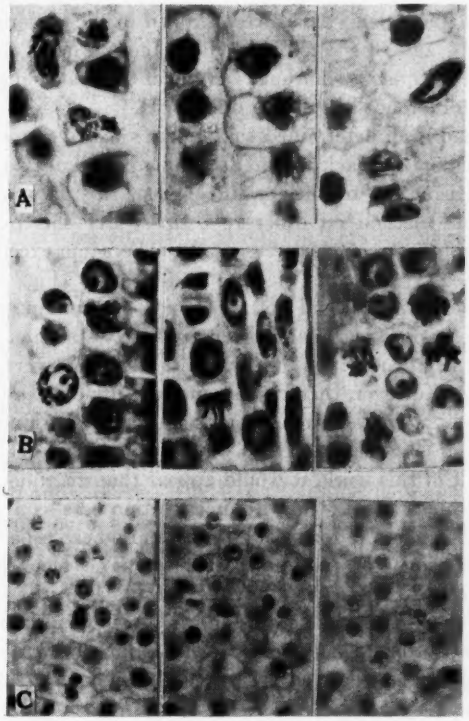

FIG. 3. Root-tips of *Vicia* (A), *Zebrina* (B), and Zea (C) fixed in Bouin's fluid and stained with 1% aqueous chlorazol black E. Results satisfactory.

aqueous solution of chlorazol black E was tried. When this solution was used, it was necessary to run the sections down to water, to leave them in the staining fluid 1 to 2 hours (preferably 2 hours altho the exact time is not important), and to run them rapidly up to xylene. This method did not give satisfactory staining with most of the fixatives, but the results obtained on root tips fixed with Bouin's killer were excellent (Fig. 2). The chromosomes and nucleoli were stained an intense black and stood out clearly against the light gray

cytoplasm and dark grey nucleus. The cell walls were, in places, rather indistinct, but they could usually be distinguished, and the mitotic figures showed up very nicely.

A further experiment was carried out to see if the hydrogen-ion concentration of the solution had any effect on the staining ability of the dye. To this end phosphate buffer salts were added to both alcoholic and aqueous solutions of the dye, giving a series of dye solutions ranging from pH 5.2 to pH 9.0. Root tips killed in CRAF, Bouin's, and Benda's fixatives were stained in the buffered alcoholic dye solutions, and tips killed in Bouin's and Carnoy's were stained in the buffered aqueous solutions. None of the material showed any appreciable difference in staining due to pH.

After it had been determined that Bouin's fixative, a 1% aqueous solution of chlorazol black E, and a staining period of two hours gave the most satisfactory results for *Allium* root tips, it was decided to try the same method on other root tips. For this purpose *Zea*, *Vicia* and *Zebrina* root tips were used. The same excellent results were obtained with *Vicia* (Fig. 3a) and Zebrina (Fig. 3b) as with *Allium*. The *Zea* roots did not show such outstanding results, but this was due to poor material rather than faulty staining. The roots were harvested at the wrong time with the result that very few figures were found. However, there were some cells dividing in the extreme tip of the root which showed chromosomes as well stained as in the other material (Fig. 3c).

As a result of this work, it would appear that an aqueous solution of chlorazol black E following Bouin's fixation acts as a very simple and satisfactory stain for root-tip chromosomes, especially when preparing sections for photomicrography. The alcoholic solution recommended by Darrow and by Levine and Morrill is doubtless preferable for general histological and cytological staining, but it is not satisfactory for chromosomes. The staining abilities of neither solution appear to be effected by hydrogen-ion concentration.

The author wishes to express appreciation to Dr. L. W. Sharp and Dr. R. A. Laubengayer of the Botany Department at Cornell University for their advice and encouragement.

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NEBEL, B. R. 1940. Chlorazol black E as an aceto-carmine auxiliary stain. Stain Techn., **15**, 69.

BUFFERED ROMANOWSKY STAINING OF COLLODION COATED SECTIONS

R. D. LILLIE, *Senior Surgeon, U.S.P.H.S., Division of Pathology, National Institute of Health, Bethesda, Maryland*

It has been observed that when it is necessary to coat paraffin sections with collodion to prevent loss of parts of the sections or to prevent cell shrinkage, staining with the buffered Romanowsky technic[1] is often unsatisfactory. Under these conditions the azures generally stain well while the eosinophilic structures are tinged feebly or not at all with eosin. As the omission of accelerators had been found to give the same type of result[2], and as this defect had been remediable by increasing either the duration of staining or the concentration of dye, as well as by the use of accelerators, it was thought that increase either of staining time or of stain concentration might remedy the defect in the present instance as well.

Sections of formaldehyde-fixed tissues, either untreated or immersed in 2% collodion before or after removal of paraffin, were stained for variable time intervals in 40 ml. quantities of dilute stain containing 5 ml. acetone as accelerator, 2 ml. of a pH 3.95 citric-acid-Na_2HPO_4 buffer mixture and 2 to 5 ml. stock glycerol-methanol solution of polychrome methylene blue eosinate (lot EBKS made in our laboratory). The collodion coated sections were prepared by soaking 10 minutes in 2% collodion, draining, wiping the backs of the slides clean, hardening 10 minutes in chloroform (thus removing the paraffin if it had not already been removed), passing thru chloroform 95% alcohol mixture, into 95%, then 80% alcohol and finally water. After staining, sections were rinsed, dehydrated and decollodionized with acetone from a dropping bottle, passed thru a mix-

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[1]Lillie, R. D., and Pasternack, J. G. 1932. Romanowsky staining of tissues with buffered solutions. Arch. Path., **14**, 515; 1936. Romanowsky staining of tissues with buffered solutions. II. Current modification. J. Tech. Methods, **15**, 65, 1941.

Lillie, R. D. 1941. Romanowsky staining of tissues with buffered solutions. III. Extension of the method to Romanowsky stains in general. Stain Techn., **16**, 1; 1941. Various thiazin and fluorane dyes in paired combinations as neutral stains for tissue. J. Tech. Methods, **23**, (*in press.*)

Highman, B., and Lillie, R. D. 1943. Romanowsky staining of tissues with buffered solutions. IV. Influence of fixatives on buffer requirements. J. Tech. Methods, **23**, (*in press.*)

[2]Lillie, R. D. 1943. On the use of acetone and methanol in the Romanowsky staining of tissues. Stain Techn., **18**, 195.

ture of equal parts of acetone and xylene into two changes of xylene, and mounted in xylene clarite.

Or, one may dehydrate with two changes (30 seconds or more) of absolute iso-propyl alcohol[3] and transfer to equal parts iso-propyl alcohol and xylene, then clear in two changes of xylene; this leaves the collodion film in place, and staining results are identical with those attained by the acetone method. The collodion retains a moderate amount of blue, which does not seem to interefere greatly with study of the sections. If the sections do not clear in the first xylene, they may be carried back thru the mixture into the second iso-propyl alcohol for another half minute, and then cleared as before.

Results: Fully satisfactory staining of sections not treated with collodion was attained in one hour with 2 or 3 ml. stock dye to 40 ml. mixture, in 30 minutes with 4 ml. and in 15 minutes with 5 ml. Sections coated with collodion after removal of paraffin gave similar staining in 2 hours with 2 or 3 ml. stock stain, in one hour with 4 ml. and in 30 minutes with 5 ml. Coating with collodion before removal of paraffin probably gave thicker films. Staining was slightly slower and slightly inferior at the shorter time intervals, while optimal staining was not attained in less than one hour even with 5 ml. stock stain. The practice of coating with collodion before removal of paraffin also seemed less satisfactory in holding smooth muscle in place.

Conclusion: Increasing either the stain concentration or the staining interval by 2–2.5 times will give satisfactory staining of collodion coated sections by the buffered Romanowsky technic. Iso-propyl alcohol is a satisfactory dehydrating agent for collodion-coated sections stained with Romanowsky stains.

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[3]Romeis, B. 1932. Taschenbuch der mikroskopischen Technik. *13th Ed.* R. Oldenbourg, München and Berlin.

NOTES ON TECHNIC

Acetone and Methanol in Romanowsky Staining of Tissues

Tissue-staining with dilute solutions of azure and methylene blue eosinates ordinarily requires overnight treatment for satisfactory color contrasts and intensities. However, the addition of acetone and methanol to the diluted staining solution will enable satisfactory staining in an hour. For many years[1] we have been using the acetone, methanol acceleration suggested by McNamara[2] with quite satisfactory results.

When a 1:80 dilution of a pooled mixed thiazin eosinate solution in methanol and glycerol aa, was used with a buffer level of pH 3.95 for formalin-fixed tissues[3]; it was found that 3 to 5 cc. acetone in 40 cc. of the final staining mixture gave satisfactory staining in one hour, as did also 8 to 10 cc. methanol or 3 to 5 cc. of each. Best were 5 cc. acetone alone, 10 cc. methanol alone and 4 cc. of each.

Since omission of these accelerators gave only the blue component in one hour, changing the buffer level to pH 3.7, 3.4 and 3.15 was tried, but this did not correct the lack of eosin staining. Increasing the stain concentration from 1:80 to 1:40, 1:20 and 1:10 gave progressive improvement of staining in progressively shorter intervals so that the 1:10 dilution gave quite acceptable staining in one hour, and very good in 2, 4, and 6 hours, even without added acetone or methanol. Also our usual mixture of 2 cc. stain in 40 cc. diluent gave more vivid staining with 5 cc. added acetone than did the 0.5 cc. stain tried first.

Concluding, the use of acetone, methanol or mixed acetone and methanol accelerators in neutral stain mixtures permits the use of smaller quantities of dye or shorter staining intervals or both than are possible without, but brilliancy of staining increases with longer time and greater dye concentration.—R. D. Lillie, *Senior Surgeon, U.S.P.H.S., Division of Pathology, National Institute of Health, Bethesda, Md.*

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[1]Lillie, R. D., and Pasternack, J. G. 1936. Romanowsky staining with buffered solutions. II. Current modification. J. Tech. Methods, **15**, 65–70.

[2]McNamara, W. L. 1933. Giemsa stain for tissue, rapid method. J. Lab. and Clin. Med., **18**, 752.

[3]Highman, B., and Lillie, R. D. 1943. Romanovsky staining with buffered solutions. IV. Influence of fixatives on buffer requirements. J. Tech. Methods, (*in press*).

The Use of Cellophane in Pollen Tube Technic

For the examination of chromosomes in pollen tube divisions, the pollen is usually sown on a surface of agar or gelatin, made up with a suitable nutrient solution of sugar. Swanson[1] in particular has described improvements in that method. Immersion of the pollen and consequent bad germination often result from sowing pollen on a fluid drop. Agar and gelatin prevent this, but are difficult to handle and particularly to mount satisfactorily.

We have found that the use of a small square piece of sheet cellophane entirely overcomes these difficulties. A small square of cellophane (about 2×2 cm.) which must not be thicker than 0.04 mm. and not of the waterproof type, is soaked in the solution to be used and well blotted. The solution is a sugar solution of suitable strength, e.g. 8% cane sugar for *Tradescantia*. Drugs such as colchicine, as recommended by Swanson for spreading the chromosomes, can be added. A large drop of the solution is placed in a petri dish and the cellophane square floated on this without wetting its upper surface. Pollen is then sown on the cellophane, and the dish closed. The pollen tubes adhere to the surface, permitting the manipulation of the cellophane square with tweezers in the subsequent fixation, staining and mounting, without the need of any support. The squares can be dehydrated and mounted in Canada balsam of Euparal, making optically satisfactory permanent preparations. The method is particularly suitable for the use of the Feulgen technic, and is in fact the only simple way of using this stain with pollen tubes. Acetocarmine, orcein or other stains of that type may also be used.—L. La Cour and A. C. Fabergé, *John Innes Horticultural Institution, Merton Park, London, England.*

[1]Swanson, C. P. 1940. The use of acenaphthene in the pollen tube technic Stain Techn., 15, 49–52.

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

BOOK REVIEW

MUNOZ, F. J. and CHARIPPER, H. A. **The Microscope and Its Use.** 5½ x 8½ in. 334 pp. Cloth. 154 illustrations. Chemical Publishing Co., Brooklyn, N. Y. 1943. $2.50.

A review has appeared elsewhere in these pages (Stain Techn., **18**, 41, 1943) of a book by the senior author, having the same title, but in Spanish. The present volume seems to have been intended originally as a translation of the Spanish book; but in the course of preparation has developed into something more comprehensive. It still treats the subject in the same way, however, simply enough for the beginner but in sufficient detail to be valuable even to those who have used the microscope for years.

Little needs to be added here to the review of the Spanish edition. The chief additions which the authors have made are: a 40-page chapter on the microtome; an expansion of the chapter on the polarizing microscope, with numerous figures to illustrate the principles involved; an increase in the total number of illustrations from 82 to 154.

The authors state in the preface that "this book is not intended as a scientific treatise but as a guide to aid technicians and students in the use of the instrument." Altho this is true, the book is a valuable contribution, if for no other reason, because the authors combine the experience of one in the field of optical manufacture and of the other in teaching microscopy.—*H. J. Conn.*

MICROTECHNIC IN GENERAL

BOURDON, P. **Remplacement des lamelles couvre-objet par un vernis cellulosique dans le montage des preparations.** *Bull d'Histol. Appl.*, **18**, 238–40. 1941.

Glass cover slips may be replaced by a transparent cellulose lacquer such as that used for nail polish. The method used is as follows: Run the section up to 95% alcohol, blot lightly with filter paper, and dehydrate with 1 or 2 drops of absolute alcohol. With a fine brush apply a drop of lacquer large enough to cover the whole preparation. Dry 24 hr. The slide may be examined in the usual manner except that paraffin oil must be used instead of the ordinary immersion oil.

The outstanding advantage of this method is its simplicity, with no cover slips and no Canada balsam required. Furthermore, the preparation is mounted in a very solid medium so that no displacement can be caused by pressure on the section. The disadvantages are that one must wait 24 hr. before examining and that it is impossible to use high dry objectives which are corrected for the thickness of a cover slip. The latter objection, however, may be overcome by placing a cover slip over the section while it is being examined. If the lacquer does not form a uniform layer over the section, it may be repaired by adding a few drops of absolute alcohol. After drying for about an hour, the preparation may be covered with a new layer of lacquer.—*Jean E. Conn.*

ELMES, B. G. T. **Transmission of histological sections by air mail.** *J. Path. & Bact.*, **55**, 228. 1943.

Medical officers in the tropics frequently require the services of a consultant pathologist; this coöperation despite isolation can be readily arranged by sending sections by air mail if they are prepared as follows: Fix sections to mica sheets in the usual manner and cut strips of suitable size for enclosure in envelopes. Dip

the sheets in melted paraffin; in the tropics use a high melting point paraffin (58–60° C.) both for embedding and coating. The strips will stand considerable bending without damage and can be sent, either stained or unstained, at the usual air-mail rate.—*S. H. Hutner.*

PILLET, D. **Confection des coupes à la paraffine pendant la saison chaude.** *Bull. d'Histol. Appl.*, **18**, 267. 1941.

To avoid difficulty in sectioning paraffin blocks during hot weather, the author suggests keeping the microtome blade and even the microtome itself in the refrigerator until time for use. The blocks of paraffin should be mounted several hours in advance and kept in the refrigerator until ready to cut. Slides with a drop of ice water on them are placed on top of drinking glasses filled with pieces of ice. As the ribbons are cut, they are placed on the slides and are unfolded in the usual manner, using a hot plate for the final step. By this very simple technic sections of 5μ may be cut at temperatures as high as 30–32° C., using paraffin with a melting point of 54–56° C.—*Jean E. Conn.*

SMITH, G. STEWART. **A danger attending the use of ammoniacal solutions of silver.** *J. Path. & Bact.*, **55**, 227–8. 1943.

Several laboratory explosions provide proof that ammoniacal solutions of silver become explosive on standing and therefore should never be stored.—*S. H. Hutner.*

DYES AND THEIR BIOLOGICAL USES

DONIACH, J., MOTTRAM, J. C., and WEIGERT, F. **The fluorescence of 3:4 benzopyrene in vivo. Part I: The distribution of fluorescence at various sites, especially the skin of mice.** *Brit. J. Exp. Path.*, **24**, 1–9. 1943.

The use of fluorescence spectroscopy and microscopical fluorescence observations to study the fate of benzopyrene in living animals is reported. After inoculation of benzopyrene into mice and rabbits the fluorescence of the kidney, lung and liver changes slowly within 2–4 hr. from the violet color characteristic of dissolved benzopyrene to a new blue fluorescence. The milk of lactating mice shows the first signs of blue fluorescence about 4 hr. after inoculation. The clearest effects may be seen after painting the skin of white mice with benzopyrene solution; the fluorescence, at first violet, changes to blue about 6 hr. after painting, increasing in strength until the 4th to 6th day, and fading slowly into the natural fluorescence of the skin within 3 weeks. In the kidney the blue fluorescence is confined to the cortical zone. In the skin of painted mice under the microscope it is seen most strongly in the rows of the hair-bulbs. The blue-fluorescent compound is produced locally in the painted area of the skin, and is not deposited there from the blood-stream carrying benzopyrene or any derivative of benzopyrene. It has acid properties, and is soluble in ether and in dilute alkali. Its persistence in the skin over a period of several weeks after one single painting suggests that the blue-fluorescent derivative of benzopyrene is more closely connected with carcinogenesis than the parent hydrocarbon itself, which disappears from the skin within two days. As further evidence, tumors can be produced by benzopyrene in the lung, liver and mammae (tho not in the kidney cortex), which are just those sites where the blue-fluorescent compound appears.—*J. A. Kennedy.*

DONIACH, J., MOTTRAM, J. C., and WEIGERT, F. **The fluorescence of 3,4-benzopyrene in vivo. Part II: The inter-relationship of the derivatives formed in various sites.** *Brit. J. Exp. Path.*, **24**, 9–14. 1943.

Mice were fed and inoculated intravenously with benzopyrene, and an attempt was made to trace the origin of the blue fluorescence which appeared in the lungs, liver and kidneys. It was found to be due to locally changed benzopyrene, not to "bile—BPX" reabsorbed from the intestines.—*J. A. Kennedy.*

HUNTER, JAMES H., and KOLLOFF, H. G. **Sulfanilamide Compounds. VII. Thiazole Derivatives.** *J. Amer. Chem. Soc.*, **65**, 156. 1943.

Since certain sulfanilamidothiazoles have proved efficacious as antibacterial agents, a series of sulfanilyl derivatives have been investigated for the purpose of comparison. Preliminary biologic evaluation of these thiazolidine and thiazoli-

done derivatives is incomplete. On the basis of the data available, the compounds possess a higher degree of activity against experimental β-hemolytic streptococcal infections than they do against Type I pneumococcal infections. Of the compounds thus far tested against experimental infections in mice, 3-(*p*-nitrobenzenesulfonyl)-2-thiazolidone and its 4- or 5-methyl substituted derivatives have shown moderate to good activity against β-hemolytic streptococcal infection, altho their antipneumococcal activity is but slight; and in general these derivatives possess much more activity than do the corresponding 3-sulfanilyl-2-thiazolidones.—*A. P. Bradshaw.*

ANIMAL MICROTECHNIC

CORNBLEET, THEODORE, and POPPER, HANS L. **Properties of human skin revealed by fluorescence microscopy III. Paraffinoma.** *Arch. Dermat. & Syph.*, **47**, 637–9. 1943.

The paraffin lakes in sections of tissue from paraffinomas examined under the fluorescence microscope are seen to emit a vivid turquoise blue color. The elastic tissue is hypertrophied (the individual fibers) and imparts its usual brilliant color.—*J. A. Kennedy.*

KALTER, S. S. **A quadruple staining method for tissues.** *J. Lab. & Clin. Med.*, **28**, 995–7. 1943.

The following method is a development of Fleming's triple stain: Fix and dehydrate the tissue by any preferred method. If Bouin's solution (preferred) is used, remove picric acid with a few drops of Li_2CO_3 solution in the 70% alcohol dehydration series. If Zenker's fluid is used, remove the $HgCl_2$. Embed the tissue in paraffin and cut at 7.5μ. Dry slides overnight instead of using an albumin fixative which will cloud the stain. Run the slides down into tap water. Stain 24 hr. in safranin O (Safranin O, 0.2 g.; formalin, 4.0 ml.; sodium acetate, 0.5 g.; 50% alcohol, 100 ml.). Place in crystal violet 1–2 min. (Crystal violet 0.5 g. in 100 ml. of distilled water). Wash in tap water. Place in 50% alcohol for 2 min., then in 95% alcohol 2 min. Put in a mixture of fast green and orange II (saturate clove oil by adding 0.5 g. fast green FCF to 80 ml. of oil for 24 hr., shaking and filtering; then add 40 ml. of the filtrate to 30 ml. of clove oil similarly saturated with orange II). After 5 min. differentiate under the microscope till the connective tissue is green. Place in clove oil for 10 min., then in clove oil saturated with orange II for 10 min. Differentiate under the microscope. Put thru 2 changes of xylene of 10 min. each. Nuclei stain red, nucleoli purple or purplish red and the nuclear membrane dark red. Cellular cytoplasm is pink to red except in Henle's loop (light green). Connective tissue is green and elastic fibers yellow. Fibroblasts are green with purple nuclei. Muscle is tan and red blood cells orange. Polymorphonuclears have purple nuclei. The method is especially useful for students of histology.—*John T. Myers.*

POPPER, HANS, and LOEFFLER, ERNEST. **Fluorescent granules at the glomerular pole of human kidneys.** *Proc. Soc. Exp. Biol. & Med.*, **53**, 68–9. 1943.

Granules surrounding or invading the wall of arterioles were found by fluorescent light. In frozen sections they appeared whitish to golden brown; the arteriole was recognized by blue fluorescent fibers. Fluorescence was not changed by oxidizing or reducing agents, acid or alkali. Lipoid solvents reduced fluorescence after prolonged treatment. It was lost after embedding in paraffin. For exact localization of granules, sections were slightly stained with hemalum which does not interfere with fluorescence but permits observation with visible light.—*M. S. Marshall.*

PLANT MICROTECHNIC

BRODIE, HAROLD J. **Protoplasmic continuity in the powdery mildew Erysiphe graminis DC.** *Canad. J. Res., Sec. C*, **20**, 595–601. 1942.

The existence of septal pores and protoplasmic continuity in *Erysiphe graminis* was demonstrated by the following slight modification of Wahrlich's technic: prepare a strong solution of 3 g. iodine and 3 g. KI in 20 ml. distilled water;

fix fresh material (infected tissue) in a dilute iodine solution (10 drops of strong iodine solution and 20 ml. distilled water) for 2 hr.; rinse with distilled water; add chlor-zinc iodine (formula not given) to material on slide, heating continuously, if necessary, until steam appears; remove chlor-zinc iodine with blotting paper; rinse with water; add several drops of strong iodine solution and allow to stand 30 min.; remove strong iodine solution and rinse quickly with dilute iodine solution; mount in chlor-zinc iodine or in 30% glycerin.

For photomicrography better results were obtained by staining with a 10% aqueous nigrosin instead of using the above procedure.—*H. P. Riley.*

REEVE, R. M. **A microscopic study of the physical changes in carrots and potatoes during dehydration.** *Food Research,* **8,** 128–36. 1943.

Samples were placed in screw-cap vials and evacuated to remove as much air as possible. Impregnation with celloidin in a paraffin oven at 55° C. for at least a week followed. Hardness of the dried potato samples required trimming of the celloidin block to expose the material slightly and submerging it in glycerin-alcohol for several hours to soften the tissue, but even with this treatment only fragmentary sections could be obtained. "Some of the sections were stained with fast green in a solution of seven parts xylene to three parts ethyl alcohol, or with safranin in absolute alcohol followed by differentiation in the fast green solution. Aqueous and dilute alcohol solutions of the stains produced partial rehydration effects on the tissues and therefore were not used. After staining, the sections were washed in alcohol and cleared in xylene before mounting in Canada balsam. Photomicrographs were taken at various magnifications, using an orange gelatin filter to produce photographic contrast." Vegetable fats were stained with Sudan IV (method not given). The iron-adsorption method, giving a Prussian blue reaction, and ruthenium red (1:5,000 dilution) were used to demonstrate the pectins.

Steam blanching of thin slices of vegetables prior to dehydration is desirable to give more uniform rate of dehydration and uniform starch gelation, as well as enzyme inactivation and other chemical changes.—*F. E. Nelson.*

REEVE, R. M. **Microscopy of the oils and carotene bodies in dehydrated carrots.** *Food Research,* **8,** 137–45. 1943.

Fresh carrot tissue was sectioned on freezing and sliding microtomes. After reconstitution by cooking, dehydrated samples were sectioned on the freezing microtome; some unreconstituted samples were cut on the sliding microtome. Treatment with Fleming's solution to preserve the lipoids, followed by preparation by the paraffin method for sectioning on the rotary microtome, was used on both fresh and dehydrated carrots. Paraffined sections were stained and mounted in Canada balsam. Other types of preparation were stained and mounted in water, glycerin or glycerin jelly. Delafield's stain was used for cell walls and cytoplasmic staining. Sudan IV was used as a fat stain. The polarizing microscope was used in some studies of fat and carotene distribution.—*F. E. Nelson.*

REEVE, R. M. **Changes in tissue composition in dehydration of certain fleshy root vegetables.** *Food Research,* **8,** 146–55. 1943.

Certain changes occuring during dehydration were studied histologically as follows: Fresh tissues were sectioned in both the fresh condition and after embedding in paraffin. The reconstituted dehydrated foods were sectioned on the freezing microtome. Dehydrated samples were sectioned after being embedded in celloidin. Pectins were stained by the ruthenium red method of Branfoot and oil globules were stained with Sudan IV. The author concludes that blanching increases oil protection of the carotene and gels the starch to give protection to both oils and carotene.—*F. E. Nelson.*

SHEFFIELD, F. M. L. **The 'blotches' on leaves of Arran Pilot potatoes.** *Ann. Appl. Biol.,* **29,** 341–5. 1942.

About flowering time a grayish green blotch appears on some of the leaves of Arran Pilot potatoes. The epidermis dies and the palisade cells proliferate to form several layers of small, colorless cells. The blotching is probably of genetic origin. Histological examination was made by cutting the leaflets into strips of about 8 x 3 mm.; they were left for 1 min. in Carnoy's and fixed in Zenker's; air

was exhausted during fixation; material was washed, dehydrated, cleared, and embedded in paraffin; sectioning was at 6, 8, or 10μ according to the stage of development of the blotch; the most successful stains were safranin and light green or Heidenhain's iron alum hematoxylin, with or without a counterstain.—*H. P. Riley.*

MICROÖRGANISMS

COX, OSCAR F., and McDERMOTT, MARY V. **Methods of transmitting material to a laboratory for gonococcus cultural studies.** *Amer. J. Pub. Health*, **33**, 149–52. 1943.

The medium is prepared as follows: Mix equal amounts of sterile distilled water and defibrinated horse blood. Then add sufficient 1% aqueous crystal violet to make a dilution of 1:30,000. (Dye used was from National Aniline and Chemical Company; approx. 84% dye content.) To every 100 ml. blood-dye solution add 5 mg. para-amino-benzoic acid. Use test tubes 6 x 50 mm. with cork stoppers to fit (No. OOOXXX). Autoclave the tubes which are to contain the blood-dye solution at 15 lb. for 20 min. Fill the tubes about two-thirds full (0.6 ml.) with the blood-dye solution from a burette. Plug with cork stoppers which have previously been dipped in melted paraffin and kept under 70% alcohol. Invert tubes and seal stoppers with hot paraffin. A similar number of tubes is used for keeping swabs sterile. Dip cork stoppers in melted paraffin. Cut round toothpicks in half, make swabs at cut end of toothpicks, and insert pointed ends into stoppers. Place swabs attached to stoppers in test tubes and autoclave at 15 lb. for 20 min. Invert tubes and seal stoppers with hot paraffin.

The blood-dye method for transmitting exudates thru the mail is considered more efficient than the smear method in detecting the organisms in women and in men with chronic or latent infections. The authors do not recommend the routine sending of urine specimens thru the mail for gonococcus studies.—*M. W. Jennison.*

HAJNA, A. A., and PERRY, C. A. **Comparative study of presumptive and confirmative media for bacteria of the coliform group and for fecal streptococci.** *Amer. J. Pub. Health*, **33**, 550–6. 1943.

The following new "EC" medium is used at 37°C. for the presumptive test for coliform group organisms, and at 45.5°C. for confirmation of *Escherichia coli*: distilled water, 1,000 ml.; Bacto tryptose, 20 g.; NaCl, 5 g.; lactose, 5 g.; K_2HPO_4, 4 g.; KH_2PO_4, 1.5 g.; Bacto bile salts No. 3, 1.5 g. Filtration is unnecessary; no adjustment of reaction (pH 6.9) is required. Medium should be sterilized in autoclave at 15 lb. for 15 min. This medium inhibits fecal streptococci. A new "SF" (i.e. *Streptococcus fecalis?*) medium, for use at 45.5°C., is selective for fecal streptococci. This is made as follows: distilled water, 1000 ml.; Bacto tryptone, 20 g.; NaCl, 5 g.; glucose, 5 g.; K_2HPO_4, 4 g.; KH_2PO_4, 1.5 g.; sodium azide (NaN_3), 0.5 g.; brom cresol purple indicator (1.6% alcoholic solution), 2 ml. Sterilize in autoclave at 15 lb. for 15. min. This medium is particularly of value for determining certain fecal streptococci in swimming pools. Comparative data are given which indicate that these media are superior to others for the designated uses.—*M. W. Jennison.*

NICKERSON, J. T. R. **A modified little plate method for bacterial counts in vegetable freezing plants.** *Food Research*, **8**, 163–8. 1943.

The conventional little plate method was modified to use a slide with a raised center one inch square obtained either by fusing a glass of that size on a slide or by grinding down the ends of the slide. Such slides were cleaned thoroly, sterilized by dry heat in a closed metal container and placed on a surface maintained at 45° C. A 0.1 ml. portion of the dilution prepared by placing 10 g. of sample on 90 ml. of sterile water, which then was shaken thoroly, was placed on the raised portion of the slide, four drops of melted nutrient agar (33.5 g. Bacto nutrient agar to 1000 ml. distilled water) added and mixed with the sample by running a sterile wire thru the culture 15 times and spreading the material to the edge of the raised area. After incubation at 25° C. (77°F.) for 16 hr., the slides were dried on a hot plate at 80°C., treated with 1% aqueous $Fe_2(SO_4)_3$ for 20 sec., washed, treated with 0.5% aqueous hematoxylin for 15 to 30 sec., washed and dried. Colonies were counted with the 16 mm. objective of the microscope.

The results obtained by this method were found to correlate quite well with the results of agar plate counts on the same samples, incubation for 3 days at 25° C. being employed. Neither method proved highly accurate as a means of determining absolute population, but both could be of considerable worth in evaluating the condition of frozen products.—*F. E. Nelson.*

PERRIN, T. G. **A stain for Treponema pallidum.** *Amer. J. Clin. Path., Tech. Suppl.*, **7**, 28. 1943.

The procedure is as follows: Dry smears from exudate or scrapings in air; stain 2 min. with heat or 6 min. at room temperature in a mixture of distilled water 10 ml., commercial formalin 1.0 ml., acetic acid 1.0 ml., and Ziehl's fuchsin 4.0 ml.; wash by immersion in water and gentle movement back and forth; dry in air. The stain keeps well for a month.—*L. Farber.*

HISTOCHEMISTRY

GOMARI, G. **Calcification and phosphatase.** *Amer. J. Path.*, **19**, 197–209. 1943.

The author presents a modification of his method for the demonstration of phosphatases, devised for the simultaneous visualization of preformed deposits of calcium salts and sites of phosphatase activity. The tissue is fixed in 80% alcohol and embedded in paraffin. Sections, affixed to the slide, are run thru xylene and alcohols to distilled water, from which they are placed for 6–12 hr. in a 2% solution of cobalt acetate. After rinsing thoroly in distilled water, the sections are transferred for 10 min. to a dilute buffered solution of yellow "ammonium sulfide" (5 to 6 drops of sulfide to a Coplin jar full of phosphate maleate buffer of about pH 7). The sections are next rinsed in distilled water and placed in an incubating mixture (2% sodium glycerophosphate, 25 ml. 2% sodium barbital, 25 ml.; distilled water, 50 ml.; 2% $CaCl_2$, 5 ml.; 2% $MgSO_4$, 2 ml.; chloroform, a few drops; a few drops of a 1% solution of ammonium or sodium sulfide) for 5–6 hr. at 37°C. The sections are rinsed thoroly in distilled water and transferred for 15 min. to a 1% solution of $Pb(NO_3)_2$. Then the sections are rinsed in distilled water and stained for 15 min. in a mixture of a 0.5% solution of methyl green and a 0.5% solution of acridine red (Grübler). From the stain the sections are differentiated in 95 and 100% alcohols, cleared in xylene and mounted in balsam. The method stains preformed deposits of calcium, black; sites of phosphatase activity, purlish red; nuclei, green-blue—*J. O. Foley.*

WOLF, A., KABAT, E. A., and NEWMAN, W. **Histochemical studies on tissue enzymes. III. A study of the distribution of acid phosphatases with special reference to the nervous system.** *Amer. J. Path.*, **19**, 423–39. 1943.

The authors outline a method for the demonstration of acid phosphatases in sections of tissue which have been embedded in paraffin. The method is particularly applicable to sections of nervous tissue. Tissue is selected and fixed in cold absolute acetone for 24 hr., after which it is passed thru absolute alcohol and toluol, remaining in each for 24 hr. From toluol the tissue is embedded in paraffin. Sections 10μ thick, affixed to slides, are run thru xylene and absolute alcohol, washed rapidly in tap water and transferred to an incubating mixture (acetate buffer at pH 4.7, 12 ml.; $Pb(NO_3)_2$ M/10, 10 ml.; distilled water, 74 ml.; 3.2% sodium-β-glycerophosphate, 4 ml.) where they are kept at 37°C. for 19 to 24 hr. Sections adjacent to those stained for acid phosphatases are used as controls. These are placed in the incubating mixture which is modified by the addition of 0.001 to 0.01 *M* NaF which inhibits enzyme activity. Sections are removed from both incubating mixtures and washed 30 min. in 6 to 8 changes of distilled water. Then the sections are placed in a dilute solution of ammonium sulfide for 2 min., washed thoroly in tap water, rinsed in distilled water, counterstained lightly with Harris' hematoxylin and eosin, and mounted in balsam. In the finished sections the site of enzyme activity stains light brown to black depending upon the relative amounts of enzyme present, whereas such sites are uniformly negative in the control sections.—*J. O. Foley.*

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The Stain Commission is a coöperative organization affiliated with the National Research Council. It has official connection with several scientific societies, and its executive committee consists of representatives of these societies, as follows:

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Brilliant green

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Congo red

Cresyl violet

Crystal violet

Eosin, bluish

Eosin, yellowish

Erythrosin B

Ethyl eosin

Fast green FCF

Fuchsin, acid

Fuchsin, basic

Giemsa stain

Hematoxylin

Indigo carmine

Janus green B

Jenner's stain

Light green, S. F., yellowish

Malachite green

Martius yellow

Methyl green

Methyl orange

Methyl violet 2 B

Methylene blue chloride

Methylene blue thiocyanate

Methylene violet

Neutral red

Nigrosin

Nile blue A

Orange G

Orange II

Orcein

Phloxine

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In the table below is given a list of the batches of stains approved since those listed in the last number of this Journal.

STAINS CERTIFIED JUNE 1, TO AUG. 31, 1943*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Brilliant cresyl blue	CV–7	66%	Vital blood staining	June 1, 1943
Erythrosin B	LEr–4	82%	Histology; bacteriology (staining)	June 7, 1943
Eosin Y	NE–20	86%	Histology, compounding of blood stains	June 7, 1943
Hematoxylin	CH–7	....	Histology, cytology	June 11, 1943
Eosin B	NEb–10	90%	Histology	June 18, 1943
Sudan III	KY–1	93%	Fat staining	June 21, 1943
Giemsa stain	GGe–10	....	Blood staining	June 24, 1943
Fuchsin, acid	NR–15	60%	Histology; Andrade indicator	June 25, 1943
Fuchsin, basic	NF–43	94%	Histology; Feulgen reaction; bacteriology (staining; in media)	June 30, 1943
Crystal violet	LC–19	94%	Histology; cytology; bacteriology (staining; in media)	July 1, 1943
Bismarck brown Y	NN–11	59%	Histology; bacteriology (staining)	July 1, 1943
Wright's stain	CWr–17	....	Blood staining	July 6, 1943
Wright's stain	LWr–18	....	Blood staining	July 14, 1943
Fuchsin, basic	DF–8	95%	Histology; Feulgen reaction; bacteriology (staining; in media)	July 26, 1943
Toluidine blue O	NU–4	57%	Histology	July 26, 1943
Fuchsin, basic	CF–27	88%	Histology; Feulgen reaction; bacteriology (staining; in media)	July 26, 1943
Crystal violet	NC–30	94%	Histology; cytology; bacteriology (staining; in media)	Aug. 7, 1943
Methylene blue chloride	NA–25	87%	Histology; bacteriology (staining) compounding of blood stains	Aug. 7, 1943
Janus green B	NJ–11	56%	Vital blood staining	Aug. 23, 1943
Brilliant green	NBg–14	93%	Bacteriology (in media)	Aug. 25, 1943
Brilliant green	CBg–11	91%	Bacteriology (in media)	Aug. 25, 1943
Crystal violet	NC–31	93%	Histology; cytology; bacteriology (staining; in media)	Aug. 30, 1943

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

STAINING PROCEDURES

This new publication, sponsored by the Biological Stain Commission, when complete, is to be a loose-leaf laboratory hand-book, consisting of 8 or 10 leaflets, essentially as follows:

Introductory: General Methods
IA. Animal histology: General tissue
IB. Animal histology: Connective and elastic tissue
IC. Animal histology: Nervous tissue
ID. Animal histology: Blood, Bone, Fat
II. Plant Histology
IIA. Plant Cytology
IIIA. Microörganisms: In smears
IIIB. Microörganisms: In sections

The methods given in these various leaflets are to be those in actual use in the laboratories of the members of the Stain Commission. A uniform method of presenting the procedures has been adopted thruout the publication, and all formulae are expressed in a standardized form. No method is to be included in Staining Procedures until it has been submitted to the general membership of the Commission, giving everyone familiar with the sort of work for which the method is intended a chance to check it over carefully. In this way, the procedures will have the endorsement of a considerable body of biologists behind them. It is planned to keep the leaflets up to date by periodical revision.

As considerable time is needed to put procedures in this shape and to secure endorsement of so many individuals, it is not planned to publish the entire handbook at once, but to issue it, leaflet by leaflet, as the various sections are ready. After the entire set is published it is planned to revise leaflets from time to time to keep them up to date. The following is an outline of the first three leaflets which were published early this year:

INTRODUCTORY
Statement of Purpose
General methods
  Fixing fluids
  Dehydration
  De-alcoholization
  Paraffin method
  Celloidin method
Staining solutions
Concentration of solutions

IA. ANIMAL HISTOLOGY: GENERAL TISSUE
Introduction
Miscellaneous tissue stains: 12 in number
References

IB. ANIMAL HISTOLOGY: CONNECTIVE & ELASTIC TISSUE
Introduction
Mallory's procedure; and 3 modifications
Eight miscellaneous procedures
Masson's trichrome stain and one modification
Silver methods
References

Each of these three leaflets is to sell for 30c postpaid. Orders will be taken now for any one (or all three) of them.

Announcement of later leaflets will be made from time to time. The prices of the other leaflets will vary from 25c to 50c according to size.

BIOTECH PUBLICATIONS

Box 353 **Geneva, N. Y.**

JUST OUT

Leaflet I-C of STAINING PROCEDURES

entitled

NEUROLOGICAL STAINING METHODS

32 pp. Price 40c

Announcement is also made of a press-board cover designed to hold the leaflets of Staining Procedures. Eventually it is planned to put on sale a special ring-book cover for this purpose; but because of war shortages, this will have to wait. Meanwhile the press-board will sufficiently protect the leaflets.

Its price is: 35c

Leaflet I-D (Miscellaneous Methods in Animal Histology) is soon to be on sale.

Its price will be: 30c

A remittance of $1.00 will pay for the Cover with Leaflets I-C and I-D when ready.

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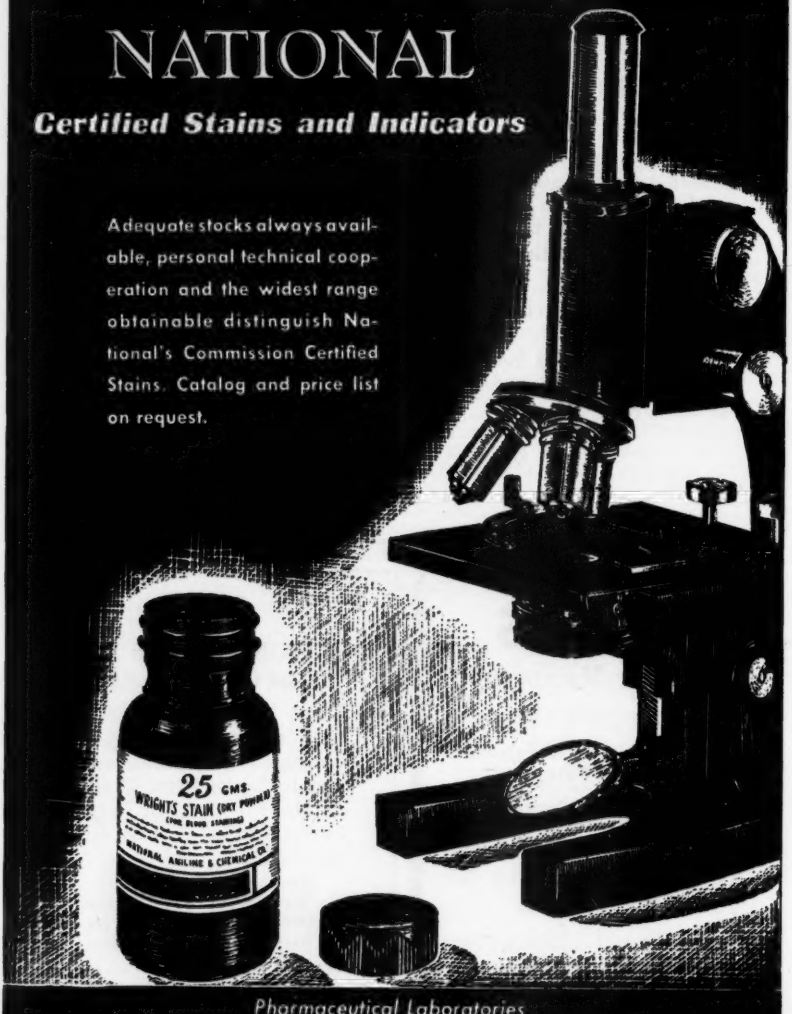

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